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LIVERPOOL SCHOOL OF TROPICAL MEDICINE

PATRON : HIS MAJESTY THE KING

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THE IMMUNITY OF MICE CURED OF TRYPANOSOME INFECTIONS

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INTRODUCTION

Since it was first shown by Ehrlich and Shiga (1904) that mice cured of trypanosome infections are refractory to reinfection by the homologous strain of parasites, a considerable amount of further work has been carried out on the subject by numerous investigators. Most of this work in mice has been done with well-known strains of *T. brucei* and *T. rhodesiense* which have been the subject of much experiment in laboratories in England and on the Continent. Similar studies with *T. congolense* in mice were not easily possible until the discovery of compounds with a pronounced curative action on infections with this particular parasite. The introduction of certain phenanthridinium derivatives (Browning, Morgan, Robb and Walls, 1938) and diamidines (Lourie and Yorke, 1939; Fulton and Yorke, 1942) has, however, rendered it possible to carry out such studies with *T. congolense*, and this has been done by Browning and Calver (1943) and by Calver (1945). We also have taken the opportunity afforded by these new compounds of making some observations on immunity in mice cured of *T. congolense* infections, whilst at the same time reinvestigating immunity phenomena in mice cured of infections with other species of trypanosome.

The strains used in these investigations were as follows:

T. rhodesiense, parent strain, maintained in mice in Liverpool for 22 years.

T. rhodesiense, atoxyl-fast strain, prepared from the parent strain in mice 16½ years ago, and maintained in mice since then.

T. rhodesiense, suramin-fast strain (suramin = antrypol, Bayer 205), prepared from the parent strain by Dr. F. Hawking in rats eight years ago, and maintained in mice since then. Resistance was enhanced seven years ago by further treatment with suramin.

T. equinum, maintained in mice for 12 years.

T. equiperdum, maintained in mice for 17 years.

T. congolense, maintained in mice in Liverpool since obtained seven and a half years ago from Dr. C. A. Hoare, who informs us that it is Strain II of Browning and Calver (1943) and Calver (1945).

These strains all produce acute fulminating infections in mice. Trypanosomes appear in the blood 1-3 days after intraperitoneal injection of moderate-sized inocula, and death follows, with the parasites swarming in the blood, a few days later, though life generally continues several days longer with *T. congolense* than with the other infections. Even when only a single trypanosome is inoculated, it was found in the case of *T. rhodesiense* that the incubation-period (i.e., the interval between inoculation and appearance of parasites in the blood) is normally not longer than five days (Lourie and O'Connor, 1937).

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The acquired resistance of the two drug-fast strains of *T. rhodesiense* was examined at the time of the observations here described, and was found to be unimpaired since last tested (Fulton and Yorke, 1941).

IMMUNITY-TESTS

Immunity-tests were carried out in the following way. Mice were infected by intraperitoneal inoculation, and two or three days later, when the peripheral blood showed about 1-10 parasites per microscope-field, they were treated by a curative dose (0.5 mgm. per 20 gm. body-weight) of either diamidino dimethyl stilbene or reduced tryparsamide thioglycollate. At varying intervals subsequently the actual tests for immunity were performed by inoculating intraperitoneally a small amount of blood (diluted in citrate-saline solution) infected with parasites of either the homologous or a heterologous strain, i.e., either the strain of the original (cured) infection or one of the other strains under investigation. At the same time control-inoculations of the same amount of infected blood were made in (a) normal mice previously uninfected and untreated, and (b) mice which had not previously been infected but which had been treated by the same drugs, and in the same dosage, as the mice whose immunity was under examination. The result of each immunity-test was one or other of the three following:

- (i) Normal infection, with normal incubation-period, signifying absence of immunity.
- (ii) No infection, indicating immunity.
- (iii) Infection after a protracted incubation-period, at least five days longer than that of control-infections. This also is evidence of immunity. Whether an immune mouse responds to inoculation in this way or by complete failure to take the infection depends partly on the number of trypanosomes inoculated, as shown by Jancsó and Jancsó (1934) and Lourie and O'Connor (1936). This has been confirmed for mice immunized against *T. congolense* in the experiment of Table I below.

TABLE I

Showing results of immunity-tests in mice cured of *T. congolense* infections 16 weeks previously, the test-inoculum containing varying numbers of homologous trypanosomes

No. of trypanosomes in test-inoculum	No. of mice		
	Became infected but incubation-period protracted	Failed to become infected	Became infected with normal incubation-period
250,000	12	3	0
5,000	3	11	1
100	0	15	0

Three groups, each of 15 mice, were reinoculated with homologous parasites 16 weeks after curative treatment of *T. congolense* infections. In the first group the number of trypanosomes reinoculated was approximately 250,000 per mouse, in the second group 5,000, and in the third group 100. The numbers of mice which became reinfected after a prolonged incubation-period, relative to those which failed to become reinfected, in the three groups, were respectively 12 : 3, 3 : 11, and 0 : 15. In only one mouse of the entire series of 45 was there apparently no immunity, in that infection ensued after a normal incubation-period.

The results of a considerable number of immunity-tests, in each of which the test-inoculum was adjusted to contain 5,000-50,000 parasites, are collected in Table II. The conclusions to be drawn, with a discussion of these conclusions, supported by further experiment, are described below under three headings, as follows: (1) tests for immunity to homologous strains; (2) tests for cross-immunity between the parent and drug-fast *T. rhodesiense* strains; and (3) tests for cross-immunity between different trypanosome species.

TABLE II

Showing results of tests in mice for immunity to the homologous and to heterologous strains of trypanosome, at varying intervals after curative treatment by diamidino dimethyl stilbene or reduced trypanamide thioglycolate, respectively

	Time since treatment (weeks)	Drug	Test-inoculum					
			<i>T. rhodesiense</i> parent strain	<i>T. rhodesiense</i> atoxyl-fast	<i>T. rhodesiense</i> suramin-fast	<i>T. equinum</i>	<i>T. equiperdum</i>	<i>T. congolense</i>
CURED INFECTIONS	4	diam.	14—			7+	7+	5+
		tryp.	24— 1d 2+	7—				
	8	diam.	2—	2—	4—			2+
		tryp.	2—	2—	2+			2+
	16	diam.	2—	2—	1— 1d 2+			2+
		tryp.	1— 1d	2—	4+			
	26	diam.	1—		2+			
		tryp.	2—	2—	2+			
	8	diam.	2—	2—	4—			2+
	16	diam.	2—	2—	2+			
	8	diam.	4—	2+	4—			
		tryp.	4+	2+	4—			
	16	diam.	1—	2+	1+			
	4	diam.	5+					11— 3d
CONTROLS	8	diam.	4+	2+				7— 10d
	12	diam.						6— 8d
	16	diam.	4+					11— 3d 1+
	20	diam.						3—
	4	diam.						8+
		tryp.	6+	5+	5+			2+
	8	diam.	2— 2d 1+	1d 2+	2— 3+			6+
	10	diam.			2—			
	16	diam.	3+	2+	3+			2+

Figures indicate numbers of mice.

— = failed to become infected.

d = became infected, but after delayed incubation-period, at least five days longer than normal.

+ = became infected, with normal incubation-period.

diam. = diamidino dimethyl stilbene.

tryp. = reduced trypanamide thioglycolate.

1. TESTS FOR IMMUNITY TO HOMOLOGOUS STRAINS

Immunity to the homologous parasites is shown in Table II for each of the four strains examined in this respect, i.e., the three *T. rhodesiense* strains and *T. congolense*. In 128 such tests homologous immunity was apparently absent in only four instances, i.e., in two mice four weeks after cure of the parent *T. rhodesiense*, in one mouse 16 weeks after cure of the suramin-fast *T. rhodesiense*, and in one mouse 16 weeks after cure of *T. congolense* infections.

Unequivocal immunity, as shown by failure of mice to become reinfected even with delayed incubation-periods, was demonstrated as late as 26 weeks from the date of curative treatment in the case of *T. rhodesiense* infections, and 20 weeks in the case of *T. congolense*. Such a long duration of immunity in treated mice has already been reported by Lourie and O'Connor (1936), who showed that it might last for at least eight months in the case of a strain of *T. brucei* which had been maintained in mice for three years, and for at least six and a half months with our present strain of *T. rhodesiense*, which at that time had been maintained in unbroken passage in mice for 13 years. More recently, Browning and Calver (1943) and Calver (1945) have described immunity lasting for at least 13 months after treatment in a strain of *T. congolense* which had been continuously passaged in mice for about 15 years. This long-lasting nature of the immunity in treated mice is in contrast to the transience of such immunity reported by the earlier workers (Ehrlich and Shiga, 1904; Franke, 1905; Ehrlich, 1907; Schilling, 1909; Schilling and Jaffé, 1909; Browning, 1908; Neumann, 1911; Terry, 1911). The discrepancy in this respect between our comparatively recent findings and those of the earlier workers may perhaps be considered as attributable, at least in part, to the varying stability of the antigenic composition of different trypanosome strains, or of the same strain at different periods in its history. There are at least two ways in which a labile antigenic constitution would conduce to an apparent breakdown of immunity, as follows:

(i) When a mouse is cured, and then reinoculated many weeks or months later, infection may ensue for the reason that the trypanosomes, passaged through a series of other mice in the interim between cure and reinoculation, might have altered during that time in their antigenic structure; the cured mouse may then, at the time of the test, still be immune to the original type of trypanosome, but this immunity involves no protection against the new type. Such an alteration of antigenic structure, during passage through mice, was detected by Lourie and O'Connor (1937) in their 13-year mouse-strain of *T. rhodesiense*. The change, described as 'spontaneous variation,' was estimated to occur only once in about 1,000 passages at that stage in the strain's history, and probably an unusually high resistance in an exceptional mouse contributes essentially towards its occurrence. Another example of the antigenic lability of a mouse-maintained strain is shown by Calver (1945) in *T. congolense* (e.g., her Strain I), where the trypanosomes habitually undergo immunological changes when an infection passes from the 'fastigium,' i.e., when trypanosomes are swarming in the blood, to the chronic stage of infection. No doubt different strains vary considerably in the facility with which they are able to assume new immunological characters. In strains with a relatively stable antigenic structure (or with a fairly ready tendency to revert to the original type), such as those with which we have been working, infection does *not* result in mice reinoculated many months after cure, because the trypanosomes, in their interim passage from mouse to mouse, have retained (or reverted to) the same antigenic features which they had possessed

at the time of cure, and the persisting immunity of the cured mice is accordingly able to assert itself against those trypanosomes.

(ii) A second way in which the labile nature of a strain's antigenic structure may give rise to an apparent breakdown of immunity is in the facility with which such a strain is able to establish itself in the hostile environment which it encounters within the immune mouse, by acquiring an altered, antiserum-fast character.

This conception of the immune state in cured mice shifts the emphasis from a consideration of the mouse's powers of acquiring and retaining an immunity towards the idea that the immunity and its duration are very largely conditioned by the stability of the trypanosome's antigenic constitution. The conception is fortified by the following observations.

A comparison of the tests for homologous immunity in mice cured of *T. rhodesiense* with those cured of *T. congolense* infections (see Table II) shows that the numbers of mice which failed to become infected, relative to those in which infection ensued only after a prolonged incubation-period, were 48:2 for *T. rhodesiense* and 38:24 for *T. congolense*. This might lead to the superficial conclusion that the mouse has greater powers of acquiring immunity to *T. rhodesiense* than to *T. congolense*. That conclusion is, however, not supported by the results of a rough *in vitro* titration of immune-body (trypanolysin) in the serum of mice at intervals after cure of infections by *T. rhodesiense* and *T. congolense* respectively, as described below.

Six mice infected with *T. rhodesiense* and four with *T. congolense* were treated two or three days later by curative doses of reduced tryparsamide thioglycollate and diamidino dimethyl stilbene respectively. Immune sera were then obtained two, three, four and five weeks later, from about 0.15 ml. blood taken from the tail of each mouse.

The quantitative tests for trypanolysis, with each immune serum, were performed by placing drops of fresh unheated guinea-pig serum, 0.075 ml. per drop, on one or two microscope slides. Dilutions of immune serum, 1/4, 1/16, 1/64, etc., were then made by mixing 0.025 ml. of the immune serum with the first drop of guinea-pig serum, transferring 0.025 ml. of the mixture to the next drop, and so on. Each drop was then sown with 0.0025 ml. of a suspension of homologous trypanosomes. This suspension had previously been obtained by mixing the heart's blood of a heavily infected mouse with a small amount of isotonic sodium citrate-saline solution, centrifuging to remove the red cells, and adjusting the trypanosome-density, by diluting with mouse serum, to about 7,500 per c.mm. (so that the eventual density of trypanosomes in each drop of diluted immune serum, in the actual tests for trypanolysis, should be about 250 per c.mm.).

The slides were then kept at 37° C. for 25-30 minutes, after which each drop was examined in a haemocytometer cell, and a count made of the number of moving trypanosomes per c.mm.

The end-point was the highest dilution in which all or nearly all the trypanosomes were lysed or motionless. Occasionally, sluggishly moving trypanosomes were present in the first few dilutions of a particular immune serum, but (with sera of high titre) not in the adjoining, higher dilutions, in which the organisms were all lysed or motionless. This 'zone' effect is presumably attributable to the anticomplementary property of mouse serum (Lourie and O'Connor, 1937), and in immune sera of low titre (4 or 16) it made for difficulty in determining the end-point. In such cases, however, the end-point was accepted as 4 or 16, if trypanosomes were reduced in number and moving sluggishly, instead of with normal activity, at these dilutions of immune serum.

Table III below shows the results of the *in vitro* trypanolysis tests. It may be seen that, measured by this method, the content of antibody against homologous trypanosomes was generally higher in mice immunized to *T. congolense* than in those immunized to *T. rhodesiense*. This is in spite of the fact that, as pointed out above, as many as 24 of 62 mice cured of *T. congolense* became infected again on reinoculation (though with prolonged incubation-periods), whereas only two of 50 mice cured of *T. rhodesiense* became reinfected under the same conditions. This strengthens the view, then, that the apparent breakdown of immunity in reinoculated mice is not to be attributed primarily to deficient

powers of immunity on the part of the host. The explanation presumably lies rather in the facility with which the trypanosome is able to alter its immunological type, and so to reinfect a mouse which remains fully possessed of its immunity to the original trypanosome-type; the further conclusion then follows that our strain of *T. congolense* is antigenically more labile than our *T. rhodesiense*, enabling it more readily to re-establish itself in an immune mouse.

TABLE III

Showing trypanolytic titres of mouse immune sera against homologous trypanosomes (*T. rhodesiense* and *T. congolense* respectively) at intervals after curative treatment

Infection	Mouse no.	Weeks after curative treatment			
		2	3	4	5
<i>T. rhodesiense</i> ...	1	4	<4	<4	Not examined
	2	64	4-16	4	"
	3	64	16	16	"
	4	4	4	4	"
	5			16	"
	6			4	"
<i>T. congolense</i> ...	7	64	64	16	16
	8	256	256	64	64
	9	256	264	16-64	64
	10	256	256	64	64

The conception of the antigenic lability of a trypanosome strain is well substantiated in the fact, repeatedly confirmed since first demonstrated by Franke (1905) and Ehrlich (1909), that the trypanosomes of a relapse usually differ in immunological type from the originally infecting trypanosome. In order to demonstrate that it is because of this property of antigenic lability that a trypanosome strain is able to reinfect an immune mouse, we performed the following experiment.

Two mice, A and B, were immunized to *T. congolense* by curative treatment. About 16 weeks later Mouse A was reinoculated with parasites of the homologous 'passage' strain (i.e., the strain maintained exclusively by inoculation from normal non-immune to normal non-immune mouse as soon as parasites become numerous in the blood). Infection resulted after the protracted incubation-period of six days.

Immune serum from Mouse B was then tested by the *in vitro* technique described above, for its trypanolytic power against (i) the homologous 'passage' strain, and (ii) trypanosomes from the successful reinfection of Mouse A. The result was lysis (titre 64) of (i) but no effect on (ii).

The homologous trypanosomes had evidently altered their antigenic character, consequent on reinoculation into immune Mouse A, and had thus been able to establish a new infection in that mouse.

It is this antigenic lability of trypanosome strains which partly accounts for the well-known difficulty of successfully immunizing men or beasts against trypanosomiasis in the field. A solid immunity against a particular antigenic type of trypanosome must be worth very little not only in the face of the considerable (if not vast) multiplicity of antigenic types in nature, but also in view of the fact that each of these types may have the capacity to change in such a way as to enable the trypanosome to thrive in an animal immunized against that type.

As judged by the immunity-tests above, *T. congolense* appears then to be more antigenically labile than *T. rhodesiense*. It is not safe to generalize too widely from observations with these isolated strains, but, if strains of *T. congolense* in general are

similarly more labile than the trypanosomes of man in Africa, then the corollary would follow that previous infection is less likely to confer protection in *T. congolense* infections of cattle than in the infections of man. (Hornby, 1941, has recently concluded that no method of immunization is available which will enable East African domestic stock to thrive in tsetse belts.) A particularly labile antigenic constitution on the part of *T. congolense* may contribute also toward the well-known difficulty of curing infections of this parasite, since it would enable the trypanosomes, by defying the host's acquired antibodies, to reappear in force, after a non-sterilizing course of treatment, much more readily than would otherwise be the case.

2. TESTS FOR CROSS-IMMUNITY BETWEEN THE PARENT AND DRUG-FAST *T. rhodesiense* STRAINS

Table II shows complete cross-immunity between the parent *T. rhodesiense* strain and its atoxyl-fast off-shoot, but not between either of these and the suramin-fast branch of the same strain.

In some of the cross-immunity tests between the parent and the suramin-fast strains, and between the atoxyl-fast and the suramin-fast strains, there was, however, an appearance of immunity, in that no infection resulted. Inspection of the table shows, though, that this result was confined to instances where cure had been effected by diamidino dimethyl stilbene, generally not more than eight weeks, but in two instances even as much as 16 weeks, previously. Where the curative agent had been the very rapidly eliminated reduced tryparsamide thioglycollate, the absence of cross-immunity was invariably demonstrated. The anomalies, of apparent protection where none was expected, are therefore most likely to be due to the prophylactic action of diamidino dimethyl stilbene, as described by Fulton (1944), although this effect persisted somewhat longer than was anticipated. The prophylactic property of the diamidine, and the lack of this property on the part of reduced tryparsamide thioglycollate, in relation to all three strains of *T. rhodesiense*, are clearly evident in the findings with control mice in Table II, which also confirm Fulton's observation (1944) that the diamidine exercises no prophylactic effect on *T. congolense* infections.

The absence of cross-immunity between the parent *T. rhodesiense* strain and its suramin-resistant branch is not surprising. When a strain of trypanosomes is made drug-resistant by the usual methods it necessarily becomes a relapse strain, since the customary technique involves the treatment, by subcurative doses, of successive relapses; and, as mentioned in the preceding section, it has been well known, since first shown by Franke (1905) and Ehrlich (1909), that there is ordinarily no cross-immunity between a parent strain and its relapse variant.

It is therefore surprising, at first glance, that a similar lack of immunity did not appear as between the parent *T. rhodesiense* and its atoxyl-fast branch. However, earlier work here also throws some light on the matter. As mentioned above, our drug-fast strains have necessarily arisen as relapse strains, and these are usually of a different immunological type from the parent strain. It was shown, though, by Mesnil and Brimont (1909), Neumann (1911), Braun and Teichmann (1912), Rosenthal (1913), Ritz (1914), and Lourie and O'Connor (1937) that a relapse strain may nevertheless gradually revert to the parent type. Schilling (1929) likewise has shown a drug-fast strain to be immunologically the same as the parent strain. Ehrlich (1909) would interpret this as showing

that certain relapse strains, which he referred to as of the 'Binio' type, comprise individuals whose protoplasm contains two distinct groups of receptors, whereas the parent strain would be of the 'Unio' type, possessing only one of these receptor groups. In course of time the 'Binio' strain would lose one of the groups, reverting to the 'Unio' parent type. A similar interpretation is favoured by Calver (1945) to explain the immunological heterogeneity of chronic strains of *T. congolense*, which eventually revert to the parent type in the course of continued passage from mouse to mouse at the 'fastigial' stage of infection. Rosenthal (1913), Ritz (1914) and Lourie and O'Connor (1937), however, showed that relapse strains may arise in the first instance as a mixture of relapse-type individuals with others still of the parent type. In the course of passage of this mixed infection, one of the components is eventually lost, and, if it be the relapse-type trypanosomes which are thus lost, then the parent type naturally remains.

It may be noted that our suramin-fast strain was prepared, in part, during a series of passages through rats, whereas the atoxyl-fast strain was prepared exclusively in mice. It is conjectural whether this difference has contributed in any way to the fact that it is the former strain which has remained immunologically distinct from, while the latter has reverted to, the parent type.

3. TESTS FOR CROSS-IMMUNITY BETWEEN DIFFERENT TRYPANOSOME SPECIES

Table II shows that there was no cross-immunity as between different trypanosome species—*T. rhodesiense*, *T. equinum*, *T. equiperdum* and *T. congolense*. This is, of course, confirmatory of a considerable amount of published work, and calls for no further comment.

SUMMARY AND CONCLUSIONS

1. The recent introduction of compounds effective against *T. congolense* infections has afforded an opportunity of investigating the immunity of mice cured of these infections. At the same time, the immunity of mice cured of other trypanosome infections has been reinvestigated.

2. The extent to which a cured mouse is refractory to reinoculation by homologous trypanosomes depends not merely on the host's powers of acquiring and retaining an immunity, but on the stability of the trypanosomes' antigenic constitution. If this is very labile, then an immune mouse will readily become reinfected, even though its powers of immunity have remained unimpaired.

3. This antigenic lability of trypanosome strains largely accounts for the difficulty of successfully immunizing men or beasts against trypanosomiasis in the field. It would also partly account for failures of treatment, after non-sterilizing drug-dosage; the host's acquired immunity, after such treatment, is unable to complete the task of eradicating the infection, since the trypanosomes are able to reappear in a changed antigenic form, in defiance of the host's immune state. Our strain of *T. congolense* is evidently more labile, in the sense here described, than our *T. rhodesiense*.

4. There was no cross-immunity between our strain of *T. rhodesiense* and a branch which had been made resistant to suramin, but there was complete cross-immunity between the parent strain and a branch made resistant to atoxyl. This is discussed in the light of earlier work. The drug-fast branches are essentially relapse strains, and it is well known that such strains usually differ immunologically from the parent strain. Sometimes, however, the trypanosomes of a relapse strain consist of a mixture of individuals

of the relapse and the parent type, and in the course of subsequent passage from mouse to mouse those of the former type become lost, and the strain therefore reverts entirely to the parent type.

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ON COELOMOMYCES FUNGI CAUSING HIGH MORTALITY OF *ANOPHELES GAMBIAE* LARVAE IN RHODESIA

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During an investigation in March, 1941, into the breeding-places of anopheline mosquitoes at Livingstone in Northern Rhodesia, some larvae of *A. gambiae* Giles were found to be infected with a growth in the haemocoel which gave rise to the formation of a large number of ovoid yellow bodies resulting in the death of the infected larvae. Mounted specimens were sent to the West African laboratory of the Liverpool School of Tropical Medicine, where they were identified by Dr. A. J. Walker as being infestations of a fungal parasite of the genus *Coelomomyces* Keilin (1921) related to those species which in 1936-38 had been studied in anophelines in Sierra Leone (Walker, 1938). The first definitely known infections of anopheline larvae by this parasite were described by Iyengar (1935) from India.

OBSERVATIONS IN THE FIELD

Work on the parasite was continued at Livingstone during the rainy seasons up to May, 1945. Three distinct fungal types or species have been found in mosquito larvae; two of these are parasitic in larvae of anophelines, mostly *A. gambiae*, and one in larvae of *Aedes* (*Mucidus*) *scatophagoides* Theo. These three types will be referred to as *a*, *b* and *c* and are recognized chiefly by differences in their thick-walled resting sporangia. One or two larvae of *Culex* (*Culex*) *simpsoni* Theo. have been found infected with the type *a* growth, but these would appear to be rare exceptions. Certain anopheline species and all culicine species, except those mentioned above, which have been found in the infected pools appear to be immune to attacks by the fungus.

OCCURRENCE IN NATURE

On the southern side of Livingstone township there are large low-lying areas of land extending over several square miles, the soil of which is a heavy dark-brown loamy clay, locally called 'mopane' clay after a small indigenous hardwood tree which is commonly found growing in this type of soil. This clay is intermixed with sandy soil in varying proportions. The flora consists mostly of grasses and sedges interspersed with small bushy trees—*Copaifera mopane* (the Mopane, Mopani or Mupane) and species of *Acacia*.

During the rainy season many surface-pools are to be found in these areas, and it is from them that mosquito larvae infected with *Coelomomyces* have been collected. A considerable part of the low-lying clay-land is within the municipal area where an extensive drainage-scheme has been carried out and where regular oiling of pools by natives employed by the Health Department is performed during the rainy season. Investigations have therefore been confined to those areas outside or on the borders of the municipal boundaries.

Most of the infected pools are in exposed, unshaded positions and are ideal breeding-places for *Anopheles gambiae*. In one case, however, a large pool near to the Zambesi River, from which batches of infected *Aedes scatophagoides* larvae as well as infected anopheline larvae were collected each rainy season, was partially shaded during the hottest part of the day; this pool contained all three types of the fungus. Infected *A. gambiae* larvae have also been collected from pools in a stream-bed (the Little Maramba stream).

In the case of nearly all the infected pools areas of mopane clay are to be found close at hand, but it is not known if the fungus is associated only with this kind of soil. Some infected *A. gambiae* larvae were once collected from a pool at the edge of the Zambesi about a mile from the Victoria Falls, but, as water was running into the pool from the river, it is possible that they had been washed down from an infected area elsewhere.

Most of the pools are of a temporary nature and dry up frequently even in the middle of the rainy season, and the infected areas are devoid of any pools for several months during the dry season. After the dry winter, the sandy mopane-clay soil becomes very porous, and, although the rainy season begins in October and lasts until the beginning of May, there is seldom enough rain before the middle of December for water to collect in the basins which constitute the infected pools. Therefore infected larvae are only to be found during three or four months of each year.

Some infected anopheline larvae, mostly *A. gambiae*, were also collected from large ponds soon after they filled up in the beginning of January; but as these ponds became overgrown with grass, and in some cases with reeds, after three or four weeks, making them unsuitable as breeding-places of *A. gambiae*, it is not known if the infection persists throughout the rainy season.

MORTALITY OF *Anopheles gambiae* LARVAE

Although no exact tests have been made, it is apparent that the infection with consequent mortality of *A. gambiae* larvae in the infected areas is very high. The writer estimates the mortality, in pools which have been under observation from 1941 to 1945, to be as high as 95 per cent. of the larvae which hatch out during the rainy season. Of those which reach the fourth instar, at least 9 out of 10 are infected and subsequently die; for, unless infection is contracted just before pupation, it is seldom, if ever, that an infected larva is able to pupate in nature. So it is estimated that, for each infected one that survives to the fourth stage, an equal number, or more, will have perished in the first three larval stages if the infection has appeared before they are half-grown, as is usually the case. This estimate is based chiefly on the proportion of newly hatched larvae to the few surviving uninfected pupae, if any, found in the pools. The mortality, however, may be influenced to some extent by the amount and frequency of rainfall; reasons for this are given below.

APPEARANCE OF THE INFECTION IN THE LARVAE

The larvae may become infected in any stage of their growth; even the very smallest (first instar) larvae have been found to contain fungal thalli and sporangia, having contracted the infection evidently a day or two after hatching. The infection may also appear in the blood-fluid just before the larva pupates, and in this case the sporangia develop in the pupa or adult. In the majority of cases in which the infection is contracted as late as this,

the growth of sporangia has been sufficient to kill the pupa or adult, but as no regular dissections of wild anophelines have been made it is not known if the infection is ever found in surviving adults in Rhodesia as is the case in Sierra Leone (Walker, 1938) and Kenya Colony (Haddow, 1942).

When there is regular intermittent rainfall it has been found that the first specks, which will become the fungal thalli, make their appearance in the blood-fluid of *A. gambiae* larvae about a week after the pool-basins have been filled by rain, assuming that there has been a dry spell prior to this and that the basins have been filled by one or more heavy showers followed by more fine weather. Under these circumstances the larvae will become infected when three or four days old, in the late first or second stage, and large numbers of sporangia will be found in the haemocoel of those larvae which survive to the third and fourth stages. Under normal conditions in nature, it only takes two or three days for the thalli to develop into sporangia.

Once the sporangia have developed the larvae are unable to pupate, but they do in a few cases survive for several days in the fourth stage, becoming as large and sometimes even larger than normal fourth stage larvae. By this time, the haemocoel may be one solid mass of sporangia, the larvae appearing opaque and turning bright yellow, orange, or dull reddish-brown in colour—particularly noticeable in larvae of *A. gambiae* and *Aedes scatophagoides*.

When *A. gambiae* larvae containing fungal thalli of type *a* have been removed to the laboratory, they do not develop the heavy infestations of sporangia which are found in those surviving in the natural habitat. Doubtless the unnatural conditions of the laboratory are partly responsible for this, particularly the lack of sunlight; but it seems probable that continual infection of the larvae may occur in nature, as sometimes small undeveloped thalli are seen to be intermingled with the sporangia. In spite of this, however, the writer is under the impression that the infection usually appears only for a short time when the pools have reached a certain concentration as a result of evaporation; when there is no rain after the pools have first been filled, all the larvae become infected at about the same time; but when rain is continuous this is less evident. Also the infectivity seems to disappear just before the pools dry up. On rare occasions larvae, which have escaped infection when one of the larger pools is in this latter condition, may be able to reach the pupal stage if rain comes again in time to prevent complete drying up; but this does not often occur.

PROBABLE MODE OF INFECTION

It seems likely that the infecting organisms, which may be the zoospores or swarm-spores liberated from the sporangia, are able to travel through the soil-water for some distance, as the following experiment indicates.

Small pot-holes, about 18 inches in depth, were dug in the mopane-clay soil of an infected area after heavy rain, when the soil was saturated with water. Larvae of *A. gambiae*, together with water, were then transported from an uninfected habitat and put into the pot-holes; some of these larvae were also kept as controls. The larvae in the pot-holes became infected after about 48 hours. In this case the possibility cannot entirely be excluded of transmission of the infection by some other inhabitant of the soil, or even by a winged insect, as it was not possible to obtain materials for effectively gauzing over the top of the pot-holes; but it seems unlikely that it was either a winged insect or any other

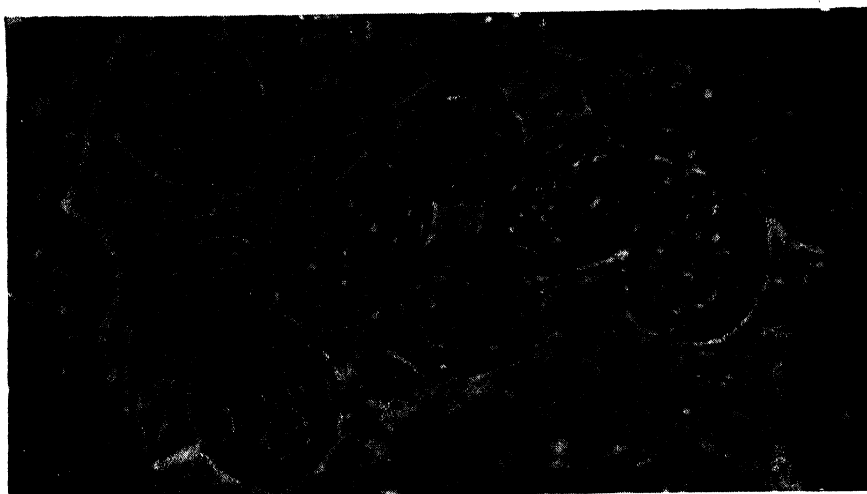


FIG. 1. A group of thick-walled resting sporangia of type *a* in a larva of *Anopheles gambiae* (in lacto-phenol; \times about 650)



FIG. 2. Thick-walled resting sporangia of type *b* in the abdomen of a larva of *Anopheles squamosus* (in Gater's fluid; \times about 210).

pool-breeding creature, as these have frequently been examined but none have ever been found to contain the fungus. It is also interesting to note that quite small puddles on native footpaths across the infected areas may have infected larvae in them if rain is sufficiently continuous to keep them filled. In one particular instance heavily infected larvae were collected from puddles on a footpath when there had not been enough rain during the previous two years for any larvae to appear in them.

NOTES ON MORPHOLOGY AND OTHER FEATURES

Owing to war-time conditions it has not yet been possible to make all the desired stained preparations, but, as details of the morphology of the genus have been dealt with principally by Keilin (1921, 1927), Iyengar (1935) and Walker (1938), general features and comparisons only are given here.

The two anopheline-infecting types, *a* and *b*, would appear to belong to group 2 of Iyengar's table, and the mucidus-infecting type *c* probably to group 1.

TYPE *a*

This is the commonest type and is chiefly parasitic in larvae of *A. gambiae* Giles, but occasionally larvae of *A. squamosus* Theo. and *A. rufipes* Gough have been found infected. A single larva of *A. rivulorum* Leeson collected from the *gambiae* pools was heavily infected, but this is the only one of the *funestus* series found to contain sporangia, although one or two larvae of *A. funestus* (type-form) from a batch which had been transported to an infected pool developed weak infections which did not mature. It therefore seems unlikely that the fungus often infects any of the *funestus* series, as is the case with *Coelomomyces africanus* Walker in Kenya (Haddow, 1942). Larvae of *A. coustani* Lav. and *A. rhodesiensis* Theo. which were collected from the pools at the same time as infected *A. gambiae* showed no sign of this infection, as also all culicine larvae except the few of *Culex simpsoni* mentioned previously. Some larvae of *A. pretoriensis* Theo. became infected when transported to an infected pool from an uninfected habitat.

Thallus: the growth consists of coenocytic thalli which are usually very short; they may be attached to and inside the tissues of the haemocoel or be carried round in the blood-fluid. Sporangia arise from the swollen thalli by budding, which may perhaps be regarded as branching of the thallus, or a single sporangium may be formed from a single piece of thallus.

Sporangia: there are two kinds of sporangia—thick-walled and thin-walled—belonging to this type and to the other two types found in mosquito larvae. The thick-walled resting sporangium (Plate. I, fig. 1) is ribbed and appears very closely to resemble that of *Coelomomyces indiana* Iyengar. There is lined sculpturing on the outside, seen under the oil-immersion lens of the microscope, similar to that illustrated by Iyengar. These sporangia vary considerably in size, as they do also in types *b* and *c*, but most are from 40 μ to 60 μ in length.

TYPE *b*

This is much rarer than type *a* and has been found in larvae of *A. gambiae*, sometimes together with type *a*, and also in larvae of *A. squamosus*.

Thallus: the growth of the thallus appears to be similar to that of type *a*, except that considerable enlargement occurs before the sporangia are formed.

Thick-walled resting sporangia (Plate I, fig. 2): these have large pits on the surface and would appear to resemble Walker's type 4. There is a line down one side (lengthwise) which is doubtless the place where the sporangium ruptures when germinating. They vary from about 50μ to 70μ in length.

TYPE *c*

This type is parasitic in larvae of *Aedes* (*Mucicus*) *scatophagoides* Theo. and so far has not been found in any other species.

Mycelium: the growth in this case is more filamentous than the other two types and more constant in width (about 7μ – 9μ); branching also occurs.

Thick-walled resting sporangia (see microphotographs—De Meillon and Muspratt, 1943): these are larger than those of types *a* and *b*, most of them varying from 60μ to 80μ in length. They seem to be somewhat similar to, but rather larger than, *C. stegomyiae* Keilin and *C. notonectae* (Bogoyavlensky). The outside surface appears smooth under the low power of the microscope but under the high power it is seen to have minute pits or pores all over. When mature, the sporangia have on occasions been seen to remain attached to the parent mycelium by a fine thread, but usually they are swept away in the blood-fluid. This attachment was observed by Iyengar in the case of the two Indian species.

TYPE *d*

A fourth type of thick-walled sporangium has been found at Livingstone which may be referred to as type *d*. These sporangia were found in the stem of a single specimen of a small sedge of the genus *Cyperus*. They are larger than type *c*, and the interior is divided into numerous cells somewhat resembling Keilin's fig. 7D (1921). They therefore probably belong to an allied species of *Coelomomyces*, but they have not been found in mosquito larvae or in any other aquatic insect or animal. There is very little evidence of disease amongst the flora of the infected areas which may be attributed to a soil fungus, and, although some grasses and other plants have been examined, no *Coelomomyces* infections have been found in them apart from the above isolated case.

GERMINATION

Germination of the thin-walled and thick-walled sporangia of types *a* and *c* has been observed. The thin-walled sporangia germinate if the larval remains containing them are left in the water from the breeding-place, liberation of the zoospores usually taking place within three to six days. The water, being turbid, has sometimes been diluted with rain-water or distilled water.

A preliminary note on the germination of the thick-walled resting sporangia of the type *c* has already been published (De Meillon and Muspratt, 1943). Professor J. S. Karling, of Columbia University, New York, has expressed the opinion (*in litt.*) that this germination process together with the kind of zoospore produced is typical of some of the Chytridiales.

Germination of the thick-walled resting sporangia of type *a* has been seen on one or two occasions and appears to be similar to the germination of type *c*, except that

extrusion of the contents of the sporangium prior to liberation of the zoospores has not been seen. Neither has it been seen in the germination of the thin-walled sporangia.

The zoospores from the type *a* thick-walled sporangia appear to be similar to those from type *c*, only rather smaller. Those from the thin-walled sporangia of both types also appear to be similar to the others, but in the latter case it has been almost impossible to wash the sporangia free from the enormous number of flagellate and other organisms living in the larval remains, or to isolate the zoospores; further study of these zoospores is therefore necessary. Occasionally a zoospore will remain in the shell of an emptied thin-walled sporangium just after the others have been liberated, and this can be studied without much danger of confusion with flagellates. Attempts to infect larvae from the zoospores produced negative results, but experiments in this line have not been absolutely exhaustive.

TECHNIQUE

In the case of the thick-walled sporangia of type *c*, two different experiments were tried, both of which led to successful germination. In the first case the larval remains containing the sporangia were dried (on a slide) two or three days after the death of the larva, when slight decomposition had begun. Slides of *Mucidus* larvae dried in this way were put in an incubator at 28° C. for two to three months before being placed in water again. In the second experiment the dead larvae were placed in water from the breeding-place, diluted with rain-water, and left in this at winter room-temperature (in Johannesburg) for three months; the sporangia were then dried and incubated dry at 28° C. for three weeks before being wetted again. In some cases water of moderate mineral-salt content (pond-water was used) was slowly evaporated down (not boiled) so as to cause a gradual increase in the mineral-salt concentration, and the sporangia were transferred or the water was changed every day or two, so that a gradual rise in the concentration was produced when they had been wetted after incubation in the dry state. It is not known if this is absolutely necessary, as germination has been induced on occasions when distilled water was used. It must also be admitted that failures have been experienced subsequently when trying to repeat the above experiments; so it is evident that all the factors involved in the germination process are not fully known.

In the laboratory, light-stimulus undoubtedly plays a part in the liberation of the zoospores. Dried thick-walled sporangia of type *c*, having been incubated and then replaced in water, were always kept in a dark incubator. When some had ruptured, after seven to nine days, they were brought out every day to be examined under the microscope. The light of the microscope lamp brought about extrusion of the contents and the liberation of the zoospores from the already ruptured sporangia, the liberation process commencing after about 15 minutes of light-stimulation. Knowledge of this considerably facilitated microphotography of the liberation process, some ruptured sporangia being transferred into a hanging drop with the aid of a micropipette.

The type *a* thick-walled sporangia did not respond to the above treatment so readily, germination being induced in only a few cases after prolonged incubation in the dry state before they were put in water again and as a result of stimulation by sunlight before and after they had ruptured.

CONCLUSIONS

The species of *Coelomomyces* referred to as type *a* which, in the pools of certain areas, destroys a high percentage of the larvae of *Anopheles gambiae* would appear to be deserving of further research by a mycologist, with a view to finding out its entire life-cycle and the possibility of using the fungus in the biological control of this malaria-carrying mosquito. The writer's privately financed investigations at Livingstone have been completed, and, as infection of laboratory-bred mosquito larvae has not yet been accomplished, he feels that it is incumbent on a public research body in Britain or southern Africa to carry on the work in the field.

Transportation of small amounts of soil to the laboratory from the infected areas—up to 100 lb. in weight when nearly dry—has been carried out. So far, the use of this soil in infection experiments has led to negative results, but it remains to be ascertained if uninfected breeding-places of *A. gambiae* in other areas could be infected by transporting to them quantities of the mopane-clay soil.

SUMMARY

1. Three species of fungus of the genus *Coelomomyces* have been found as endoparasites of certain anopheline and culicine larvae at Livingstone in Northern Rhodesia. These are referred to as types *a*, *b* and *c*.

2. A fourth species (type *d*) has been found in the stem of a sedge of the genus *Cyperus*, but has not been found in mosquito larvae.

3. In certain areas the type *a* species causes considerable destruction of the larvae of *Anopheles gambiae* Giles; the type *b* species parasitizes larvae of *A. gambiae* and *A. squamosus* Theo.; and the type *c* species parasitizes larvae of *Aedes (Mucidus) scatophagoides* Theo. Infected larvae do not pupate once the fungal growth has matured.

4. The fungi seem to be associated with a certain kind of loamy-clay soil.

5. The type *a* species would appear to be closely related to one of the Indian species (*C. indiana* Iyengar); the type *b* species is possibly similar to one of the types found in Sierra Leone (Walker's type 4); and type *c* has affinity to *C. stegomyiae* Keilin from Malaya.

6. In the laboratory the thin-walled sporangia germinated in water a few days after the death of the larvae, but the thick-walled resting sporangia have germinated only after being incubated dry at 28° C. for at least two or three weeks before being wetted again. The experimental technique is given.

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INCUBATION OF TSETSE PUPAE: INCREASED TRANSMISSION-RATE OF *TRYPANOSOMA RHODESIENSE* IN *GLOSSINA MORSITANS*

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The single strain of *Trypanosoma rhodesiense* being studied at Tinde laboratory is the one which was isolated by Corson (1936a). Its transmissibility by *Glossina morsitans* from various species of infected hosts is under investigation.

Pupae were received by post from Kondoa-Irangi in batches of 500–1,000 every week or fortnight, as the work required. The normal routine procedure was to keep them in wide-mouthed bottles closed with mosquito-netting, standing inverted on wire gauze over water in a tray in the laboratory. Flies which had emerged during the previous 24 hours were transferred to Bruce boxes, about 33 in each, for feeding on infected animals. Like the pupae, the boxes of flies were kept in trays on wire netting over water. With rare exceptions all fly-feeding was done in the morning, this work being started about 10 a.m. The flies were applied to the infected hosts daily for periods of 3–10 days, the practice being to feed them direct on the infected animal without preliminary feeding on a clean animal. Before July, 1942, the numbers of flies habitually used in experiments were about 200 on sheep, 130 on antelope and 100 on monkey. After that date the number was standardized to approximately 100 in all experiments, except sometimes for those entailing comparison or synchronous treatment, when smaller numbers had to be used.

In March, 1943, the bottles of pupae were transferred to an incubator kept at approximately 30° C. This was done in order to accelerate the emergence of clean flies. Every day at 8 a.m. the flies which had emerged in the incubator during the previous 24 hours were transferred to Bruce boxes and thereafter maintained and fed under the standard laboratory conditions as mentioned above. *No incubation was practised after the flies had entered into experiments.* Only a few observations of the relative humidity in the incubator were made. These indicated that it remained fairly constant throughout the day, and ranged between about 65 per cent. and 45 per cent. in accordance with conditions outside.

Monkeys were usually used as indicator-animals in experiments, except in those from sheep, when sheep were generally used for the purpose. When the indicator-animal was infected, showing that a transmission had been obtained, the flies were isolated singly by feeding on white rats. During the earlier period of the work numbers of flies were dissected by the modifications of Lloyd and Johnson's method described by Corson (1933) and the present writer (1936); but, in view of the frequent failure to find trypanosomes in the salivary glands on the dissection of known infected flies, this method of examination was later abandoned and all flies were isolated singly on white rats.

RESULTS

Corson (1935) wrote: 'In transmission experiments with *Glossina morsitans* and *Trypanosoma rhodesiense*, it is usually found that less than 10 per cent. of the flies, which have lived long enough, have infection of the salivary glands.'

High percentages of infected flies were rarely obtained with flies from pupae left under ordinary laboratory conditions. They were chiefly liable to occur from freshly infected reedbuck (*Redunca redunca* Pall.), the maximum figure obtained being 21·2 per cent. from this species. However, when the pupae were incubated, high percentages of infected flies became frequent. They occurred from all the species of hosts. Transmission-experiments with flies from pupae left under normal conditions were done all the year round; failure in transmission sometimes occurred, despite the fact that the host animal showed trypanosomes present in stained thick films of blood when the flies were fed. The majority of these failures occurred during the rainy season or during the cool period, June to August, which followed it. Three instances were encountered during the hottest months of the year, October and November.

TABLE I
The percentage of *Glossina morsitans* infected with *Trypanosoma rhodesiense* in flies from pupae left under normal laboratory conditions and in flies from pupae incubated at approximately 30° C.

Species of infecting host	Isolation-experiments											
	Flies from pupae left under normal laboratory conditions						Flies from pupae incubated at approximately 30° C.					
	Host	Flies					Host	Flies				
	No. used	No. of experiments	No. used	No. examined	No. positive	Percentage	No. used	No. of experiments	No. used	No. examined	No. positive	Percentage
Sheep ...	34	55	7,417	2,269	78	3·4	5	19	1,873	581	44	7·6
Reedbuck	6	22	2,326	652	43	6·8	5	13	1,274	402	59	14·7
Thomson's gazelle ...	36	72	7,891	3,062	121	4·0	6	18	1,690	610	79	13·0
Monkey ...	23	35	3,434	1,144	54	4·7	12	47	4,498	1,759	226	13·0
Total ...	99	184	21,068	7,127	296	4·2	28	97	9,335	3,352	408	12·2
Total from these host species, including results from dissection ...	136	263	30,938	10,843	349	3·2	31	108	10,428	3,700	429	11·6

Transmission-experiments with flies from incubated pupae were carried out in every month except February and November. A comparatively large number were done during the period June to August (the cooler season). Despite this, not a single failure in transmission occurred provided that the flies were fed on animals in which the blood was positive for trypanosomes.

The transmission results obtained from the four host species, sheep, Thomson's gazelle, reedbuck and monkey, with flies from normal untreated pupae and with flies from pupae which were being incubated at approximately 30° C., are shown in Table I.

The last line in the table gives the total number of infected flies obtained, including the results from dissection. The lower infection-rate shows that numbers of infected flies were being missed by dissection; and it would appear that flies in which the infection

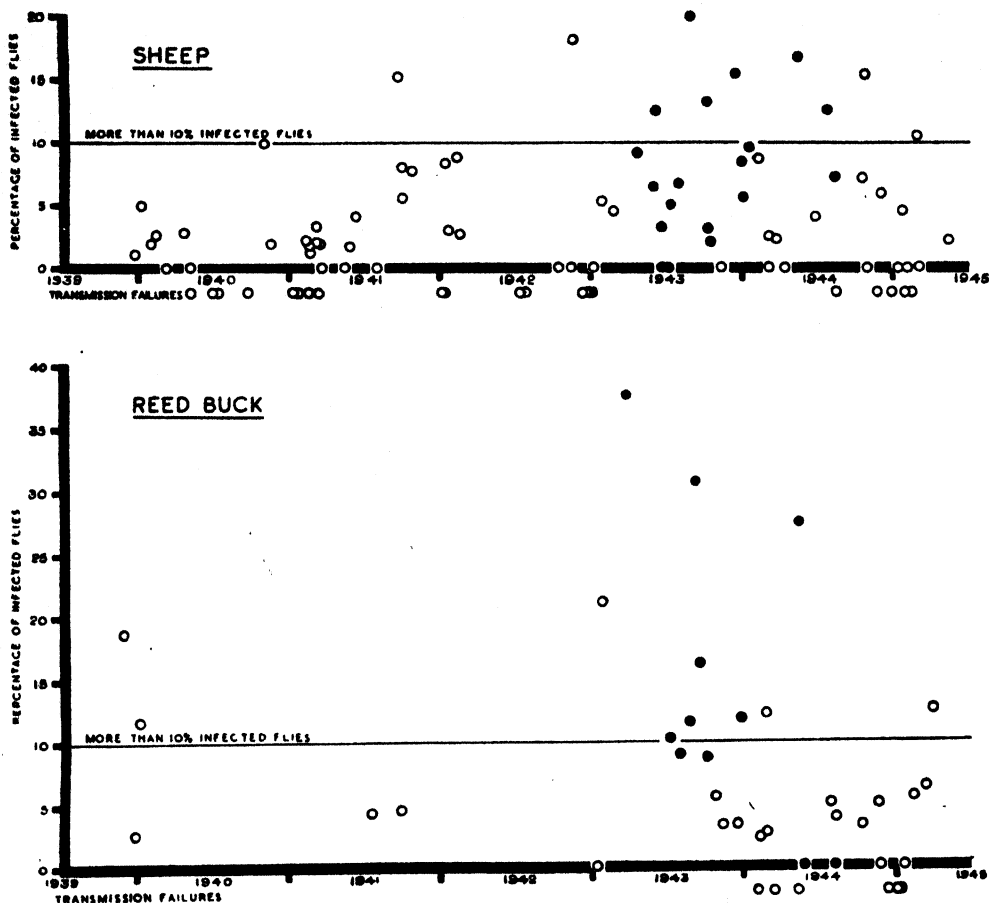


FIG. 1. Transmission-experiments with *Trypanosoma rhodesiense* in *Glossina morsitans* from sheep and reedbuck.

- = Flies from normal untreated pupae.
- = Flies from incubated pupae.

was confined to the distal deep-seated portion of the salivary glands failed to be detected by this means. The results, including the incidence of experiments in which no transmission was obtained (even after as much as 50 or more days of observation) are shown in figs. 1 and 2.* Experiments in which 10 or less than 10 flies survived to be isolated, and wherein either no flies or only a single infected fly was found, have not been shown in the figures. It was considered that such small numbers did not provide an infection-percentage of significance.

The increased transmissibility of *T. rhodesiense* in *G. morsitans* from pupae which had been incubated is obvious. The general increase must be due to a change having taken place in the flies themselves.

* Tables with details of the experiments have been submitted to the editors, who have deposited copies in the libraries of the Liverpool School of Tropical Medicine and of the London School of Hygiene and Tropical Medicine, where they are available for reference.

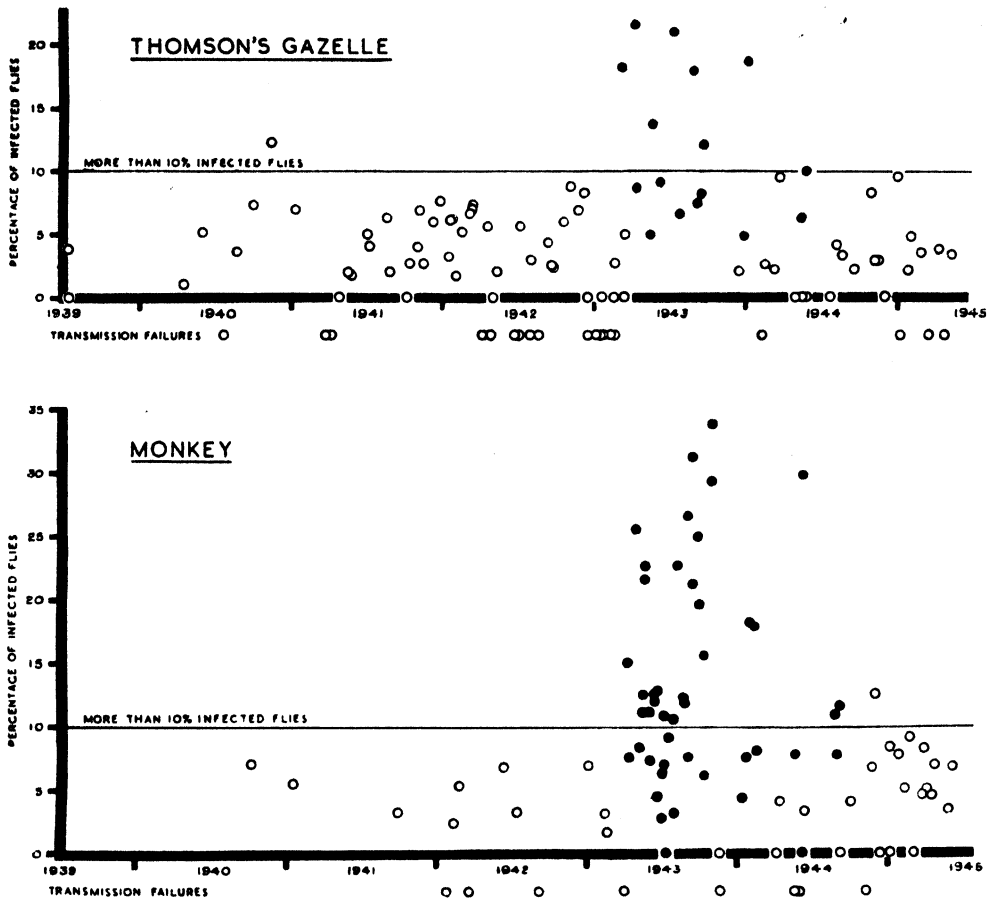


FIG. 2. Transmission-experiments with *Trypanosoma rhodesiense* in *Glossina morsitans* from Thomson's gazelle and monkey.

○ = Flies from normal untreated pupae.
● = Flies from incubated pupae.

Figs. 1 and 2, however, bring out clearly that the host animal also has an influence. By incubating the pupae, the flies fed on monkey and Thomson's gazelle gave a marked increase in transmissibility, but the increase was not so great in the flies fed on sheep or reedbuck.

In two other experiments, not shown in Table I, flies from incubated pupae were fed on a bush-pig and on a man at the time when trypanosomes were present microscopically in the blood. The infection-rates were 12.5 per cent. and 17.6 per cent. respectively, in contradistinction to the poor transmissibility obtained with flies from normal untreated pupae. It seems probable that the transmission-rate of *T. rhodesiense* from all hosts is raised when flies from incubated pupae are used, but that the amount of increased transmissibility is influenced by the host-trypanosome relationship.

Seven parallel experiments were also done, in which flies from normal pupae and flies from incubated pupae were fed concurrently on the infected host, the flies being maintained under conditions as nearly identical as possible. The results are shown

in Table II, which also records the number of days which elapsed from the first application of the flies until the indicator-animal (monkey) first showed trypanosomes in the blood.

TABLE II

Transmission-experiments carried out concurrently with flies from pupae left under normal laboratory conditions and with flies from pupae incubated at approximately 30° C.

Infecting host	Flies from pupae left under normal laboratory conditions					Flies from pupae incubated at approximately 30° C.				
	No. used	Age of flies when indicator-animal positive	No. examined	No. positive	Percentage	No. used	Age of flies when indicator-animal positive	No. examined	No. positive	Percentage
1. Monkey 593	109	No transmission (after 52 days' observation)	—	—	0	75	19 days	39	3	7.7
2. Thomson's gazelle 66 ...	95	47 days	25	0	0	89	22 "	31	2	6.4
3. Monkey 591	74	25 "	30	1	3.3	35	35 "	10	3	30.0
4. Reedbuck 17	100	33 "	26	1	3.8	100	30 "	41	0	0
5. Monkey 640	64	35 "	18	0	0	65	16 "	43	5	11.6
6. Sheep 340 ...	100	No transmission (after 57 days' observation)	—	—	0	100	30 "	14	1	7.1
7. Thomson's gazelle 66 ...	98	23 days	29	1	3.4	100	19 "	31	7	22.6
Total ...	640	Average 32.6 days	128	3	2.3	564	Average 24.4 days	209	21	10.0

It is noteworthy that, whereas two of the experiments with flies from normal pupae failed to give transmissions, transmissions were secured in every case with flies from incubated pupae. Statistical examination of the results in Table II showed that there was a significant increase in transmissibility in flies from incubated pupae as compared with flies from normal pupae ($P < 0.01$).

It will be seen that, with the exception of observation No. 3, the period taken for the indicator-animal to become blood-positive was shorter when flies from incubated pupae were used than with flies from untreated pupae. The experimental data from Thomson's gazelle, reedbuck and monkey were examined statistically to see if they showed the same trend (164 observations with flies from normal pupae and 76 from incubated pupae). Those from sheep were excluded because, as mentioned above, sheep were usually used as indicators in connection with them, whereas monkeys were habitually used for other experiments, and because sheep tend to have longer incubation-periods than monkeys. The period considered was from the date when flies were first applied to the infected host until the indicator showed trypanosomes in the blood. It was found that this was significantly shorter in the case of experiments with flies from incubated pupae than in those with flies from normal pupae ($P < 0.01$). The implication is, therefore, that incubation of the pupae tends to shorten the period needed for the completion of the developmental cycle of the trypanosomes in the fly, as well as to raise the transmission-rate.

In two further experiments (A and B) done in the relatively cool month of June, flies which had emerged under normal laboratory conditions from pupae which had recently been incubated were fed on a monkey and gave transmission-rates of 17.9 per cent. and 25.9 per cent. (7 positive in 40 and 7 in 27 flies isolated respectively). These observations are too meagre to be conclusive, but point to the effect being largely on the pupae, as distinct from the soft-bodied newly emerged flies.

Experiments A and B and the experiment on man mentioned above also provide evidence that prolongation of the incubation of the pupae further increased the transmission-rate. The flies from A were from pupae incubated only 1-2 days, while those in B were from pupae incubated 4-8 days. In the experiment on man 16 flies were used from pupae which had been incubated for 13 days. Only three survived to be isolated and two of these were infected. Of the other 42 flies from pupae incubated only one day, 14 survived and there was only one infected fly.

With regard to the question of whether other tsetse and trypanosome species behave similarly, Mr. F. L. Vanderplank, of the Tsetse Research Department, Tanganyika Territory, found in 1943, quite independently of the present writer, that the transmissibility of *T. congolense* was higher in both *G. morsitans* and *G. swynnertoni* when flies from incubated pupae were used than it was in flies from normal pupae.

With regard to the effect of incubation on the viability of pupae and flies, the records show that there is a somewhat higher mortality in incubated than in untreated pupae. Considered over similar periods in 1942 and 1943 the results were :

	Total	Dead whole puparia which remained :	
		No.	Percentage of total
Normal pupae, 1942	15,518	2,952	19.0
Incubated pupae, 1943	17,867	3,985	22.0

The higher death-rate in incubated pupae is significant ($P < 0.01$) but the difference is extremely small. Dr. Jackson, after doing the analysis, went on to point out that 1942, the year of the control pupae, had lower shade-temperature than 1943, since the rains of 1941-42 were very heavy. This might account for the difference in viability in the pupae. He thought it probable, however, that the difference was in part due to the effects of incubation. The records are not precise, since a proportion in both categories were parasitized by *Thyridanthrax* and the number of these was not noted.

It will be seen from Table I that the proportion of flies which survived to be examined from incubated pupae was, if anything, slightly higher than that of flies from untreated pupae. From the former the figure was 35.9 per cent. of those used and from the latter 33.8 per cent. The difference is significant ($P < 0.01$). The fact that the trypanosome-cycle in flies from incubated pupae tends to be shorter than in those from untreated pupae would evidently contribute to this result. The average difference was about three days.

The longevity of infected flies under laboratory conditions is shown in fig. 3. Data for flies from both normal and incubated pupae are given. All were infected from the four host species listed on Table I. There are only 244 and 234 observations of each category, since numbers of infected flies were dissected. The survival has been grouped

to the nearest five days, and the percentage of observations of any such group has been plotted against the longevity.*

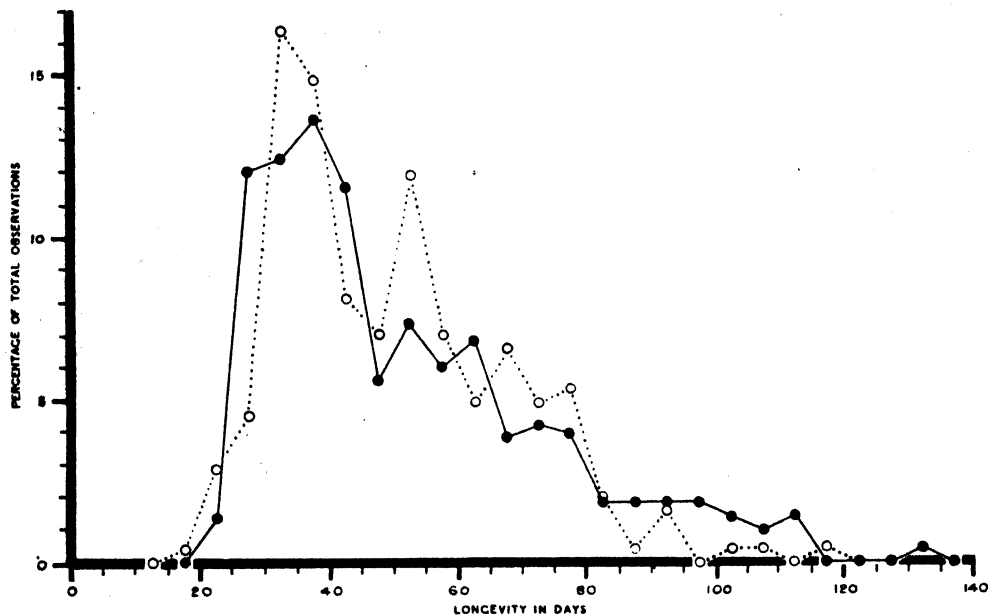


FIG. 3. The longevity, under laboratory conditions, of *Glossina morsitans* infected with *Trypanosoma rhodesiense*.

- o = Flies from normal untreated pupae.
- = Flies from incubated pupae.

Statistical examination showed that infected flies from incubated pupae survived quite as well as did those from untreated pupae.

DISCUSSION

Most of the work on transmissibility focuses attention, not on the properties of the insect vector, but rather on those of the trypanosomes, as being the primary determinant of transmissibility results.

That properties inherent in the trypanosomes may influence the infection-rate is suggested by the fact that trypanosomes from different hosts have different degrees of transmissibility. Examples are recorded by Robertson (1912), van Hoof, Henrard and Peel (1937), and Corson (1935, 1936b, 1938). It is also brought out clearly in figs. 1 and 2. Corson obtained very high rates of infection in flies fed on *Redunca arundinum* Boddaert (the southern species of reedbuck). The Bohor reedbuck (*Redunca redunca* Pall.) behaves in an analogous way, but to a lesser degree, and is the best transmitting host of the species studied in recent years at Tinde laboratory.

Another well-known explanation for transmissibility results is based on Robertson's hypothesis to the effect that the transmissibility of *T. gambiense* in *G. palpalis* depended on the condition which the parasites had reached in relation to their endogenous cycle

* See footnote to p. 20.

in the vertebrate host (Robertson, 1913). Corson (1936b) expressed the view that this hypothesis was inadequate—a view which is endorsed by the results recorded in this paper.

The fact that the infection-rate in tsetse-flies can be affected by temperature has been shown by several workers. Kinghorn, Yorke and Lloyd (1913) found that comparatively high temperatures (75°–85° F.) were needed before the cycle of development of *T. rhodesiense* in *G. morsitans* could be completed. Lloyd (1930), working with *T. brucei* and *G. tachinoides*, recorded one experiment wherein the flies were incubated between 92°–97° F. for the first eight days and then maintained at 81·2° F. for seven days, with the result that 46·6 per cent. of the flies dissected (15 in number) had salivary gland infections. However, another experiment, done under similar conditions, yielded only 8 per cent. infected flies. Taylor (1932), working with *T. gambiense* and *G. tachinoides*, showed that incubation of flies during their infecting meal raised the infection-rate, but incubation subsequent to the infecting feed merely hastened the completion of the cycle of development of the trypanosomes in the flies. Duke (1933b), in discussing the increased infection-rate in flies which had been incubated after they had fed on trypanosome-infected blood, remarked that, whereas the increased infection-rate may be partly due to the direct action of warmth on the trypanosomes, it was probably mainly due to the effect produced by the higher temperature on the vital processes of the tsetse. However, in Duke's experiments, since trypanosomes and flies were both subjected to the influence of temperature, it is naturally impossible to apportion the degree of the effect on either alone. The present work appears to afford clear-cut evidence which obviates this difficulty with regard to the tsetse.

With regard to the fly itself in relation to transmissibility, Duke (1933a), commenting on the proportion of *Glossina* infected with polymorphic trypanosomes in nature, wrote: 'In an adequate sample of wild flies it is rare to find more than 1 or 2 per thousand carrying the infective forms of the *brucei* group.' He drew attention to the fact that, despite this infrequency, flies were encountered which had double infections of a member of the *brucei* group and other trypanosome species in one and the same individual. The present writer (1942) found that, out of 105 wild *G. brevipalpis* with infected salivary glands, no fewer than 43 had double infections. He pointed out also (1945) that the results recorded by Whitnall (1932, 1934) afforded evidence of a difference in infectibility in individual *G. pallidipes*. A very significantly higher proportion of *T. brucei* infections occurred in those flies which had the salivary glands hypertrophied than occurred in normal flies. As Duke wrote (1933a), some flies are evidently outstandingly more suited for harbouring trypanosomes than others. However, whether this suitability is inherent in the fly, or is a reflection of differences in the conditions experienced by individual flies and pupae, is not known. This problem needs investigation.

The results submitted in this paper show that the susceptibility of *G. morsitans* to infection with *T. rhodesiense* can, in fact, readily be altered. Evidently the conditions experienced by the tsetse material *before* the flies come into contact with trypanosomes may have a marked effect on transmissibility results.

The following questions arise from this work:

1. Are flies, emerging from pupae which have been cooled, less susceptible to infection with trypanosomes?

2. Is the effect of temperature solely on the pupae, or has it an effect on the newly emerged flies also?

3. When pupae have been incubated, what are the anatomical or physiological reasons for an increased infectibility by trypanosomes?

4. Could the infection-rate in tsetse be raised to 100 per cent. by a combination of incubation on the pupae, temperature on the flies during their infecting feed, and a suitable host animal?

5. What are the microclimates to which tsetse pupae are exposed in the field during the year? Are the pupae subjected to considerably higher temperatures during the hot dry season, and is this the main cause of the higher percentage of infected flies found during this period?

Some observations made by the present writer (1942) at Amani, Tanganyika Territory, have a bearing on this question. He found that a relatively high proportion of wild *G. brevipalpis* (0.84 per cent. in 12,550 dissected) had salivary gland infections. The highest infection-rate occurred in September (1.12 per cent. in 3,938 flies) following the coolest period of the year. The vegetation of the Amani area is dominantly tropical evergreen rain-forest. Where the forest is broken by cultivation, a considerable proportion of deciduous vegetation is present (old Ceara rubber-plantations, etc.). *G. brevipalpis* is principally associated with such localities. It is suggested that the apparent anomaly of the highest infection-rate occurring just after the coolest period of the year may be accounted for by the fact that it is during this cool period that defoliation occurs and the ground is most liable to exposure to the sun, so that the temperature experienced by the actual pupae probably reaches its maximum.

It has been shown that, by incubation of the pupae of *G. morsitans* and *G. swynnertoni*, greatly increased transmission-rates of *T. rhodesiense* and *T. congolense* can be obtained. It remains to be seen if these results are valid for the other species of tsetse-flies and other species of trypanosomes. The subject may have considerable practical application.

SUMMARY

1. Flies from pupae which were being incubated gave a considerably higher transmissibility of *Trypanosoma rhodesiense* in *Glossina morsitans* than did flies from untreated pupae. From isolation-experiments 3,352 flies of the first-mentioned category had an infection-rate of 12.2 per cent., while 7,127 flies from untreated pupae had an infection-rate of 4.2 per cent.

2. The lower infection-rate in the total results, which include those from dissection, indicate that a considerable proportion of infected flies were not detected by dissection when modifications of Lloyd and Johnson's technique were used.

3. With flies fed on blood-positive animals, transmission failures were fairly frequent in experiments with flies from normal untreated pupae. In 110 similar experiments with flies from incubated pupae, not a single transmission failure occurred, although many of them were carried out during the cooler season.

4. The degree by which transmissibility of trypanosomes was raised varied according to the host species used. Of those studied, it was least marked in trypanosomes from sheep and most pronounced in those from *Cercopithecus* monkeys.

5. Two experiments, wherein flies were fed on a bush-pig and a man respectively,

gave infection-rates of 12·5 per cent. and 17·6 per cent. The probability is that transmissibility of *T. rhodesiense* is raised from all hosts when flies from incubated pupae are used.

6. Seven observations were made wherein flies of the two categories were fed concurrently on the infected host and subsequently maintained as far as possible under identical conditions. Transmissions were obtained in all the experiments with flies from incubated pupae, and the infection-rate was 10·0 per cent. Two failures in transmission occurred in the experiments with flies from normal pupae, the infection-rate being 2·3 per cent. in the remainder. The difference is significant. The duration of the trypanosome-cycle tended to be shorter in flies from incubated pupae than in flies from normal pupae.

7. The indications are that the effect is mainly on the tsetse pupae and not on the soft-bodied young flies when they emerge.

8. Transmissibility seems to be further raised if incubation of pupae is prolonged.

9. Incubated pupae were slightly less viable than normal pupae. The data, however, are not precise, since the proportion parasitized by *Thyridanthrax* was not noted.

10. The proportion of flies which survived to be examined from incubated pupae was significantly higher than that from normal pupae.

11. The longevity of infected flies from the two categories was about the same.

12. Factors influencing transmissibility of trypanosomes in tsetse-flies are considered. Most previous experimental work focuses attention on the trypanosomes rather than on the insect vector as determining transmissibility results. It is evident that properties may vary in both and that both play an important rôle.

13. Mr. Vanderplank's independent observations showed that the effect is manifested in *G. swynnertoni* and *G. morsitans* infected with *T. congolense*. This supports the view that the principle entailed is applicable to transmissibility of trypanosomes in tsetse-flies generally.

The work done so far shows that the conditions experienced by the tsetse pupae (and possibly the young flies) *before* they come into contact with trypanosomes may determine subsequent transmissibility.

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THE SERUM ALBUMIN AND GLOBULIN LEVELS IN AFRICANS AS COMPARED WITH EUROPEANS; WITH A NOTE ON TECHNIQUE

BY

A. F. MOHUN, MAJOR, R.A.M.C.

(Received for publication October 10th, 1945)

The serum proteins of 30 healthy young adult African males serving with the Army in the Gold Coast area have been determined by the method of Pillemer and Hutchinson (1945). A parallel series of 30 estimations was made on European Army personnel in the area. These Europeans had been in West Africa for from 1 to 15 months.

TECHNIQUE

1. *Separation of Albumin and Globulin*

Prior to the publication of the method used in this short survey, neutral salt fractionation was the method in general use for separation of globulin.

Pillemer and Hutchinson (1945) described a technique of precipitation involving the use of methyl alcohol (methanol) at a temperature between 0° and 1° C. and pH 6.7-6.9, which gave a separation of albumin and globulin agreeing well with electrophoretic determinations. These authors summarize the objections to the neutral salt method; their paper should be consulted for references to the evidence cited by various workers, as well as for details of the cold-methanol technique. Further evidence in support of the superiority of the cold-methanol technique is afforded indirectly by the observation of Cohn (1945) that, in general, albumin accounts for 58 per cent. of the plasma protein (American figures). If some 4 per cent. be allocated to fibrinogen, then the albumin/globulin ratio is 58/38, or 1.5.

The observed European albumin/globulin ratio in the present series is 1.7, which is in reasonably good agreement with the American figure.

Dole (1944) found that the albumin/globulin ratios given by electrophoretic methods are approximately two-thirds the values given by neutral salt fractionation. If, therefore, the values given by the cold-methanol technique are in good agreement with electrophoretic determinations, they should compare similarly with neutral salt fractionation values.

Jamieson (1945), employing the neutral salt fractionation technique on normal African sera, found (on 60 cases) an average albumin/globulin ratio of 1.5. The value 0.93 found in the present series is therefore in good agreement with expectation, and justifies the assumption that the albumin/globulin ratios found in the present series are close to the true values.

One point of technique requires mention. Pillemer and Hutchinson (1945) describe two alternative methods of separating the globulin-free solution from the precipitated globulin suspended therein. The first requires a refrigerated centrifuge in which the temperature does not rise above 2° C. The second entails filtration through a fluted Whatman no. 42 filter-paper, the funnel and paper having been thoroughly chilled previously. Filtration proceeds at room-temperature for 5-7 minutes, during which time the tempera-

ture of the globulin suspension does not rise above 7° C. This latter alternative is not applicable to tropical conditions. The simple arrangement shown in fig. 1 enables filtration to proceed for 30 minutes or more whilst the temperature of the globulin suspension remains below 2° C., thus avoiding any risk of alteration in the amount of protein remaining in solution. Even in conditions of extreme humidity no trouble has arisen from condensation of atmospheric moisture upon the funnel; this is presumably due to restriction of air-movement within the beaker. At the temperature operating, filtration of some 3 ml. may occupy 20–30 minutes; specific gravity measurements on the filtrate indicate that concentration due to evaporation of the filtrate is not detectable by a technique which is capable of disclosing changes in specific gravity of the order of 0.0005. This was achieved by comparing the specific gravity of the filtrate with that of graded copper sulphate solutions (Phillips *et al.*, 1944–45) by means of an adjustable non-water-miscible intermediary consisting of a mixture of chloroform and benzene.

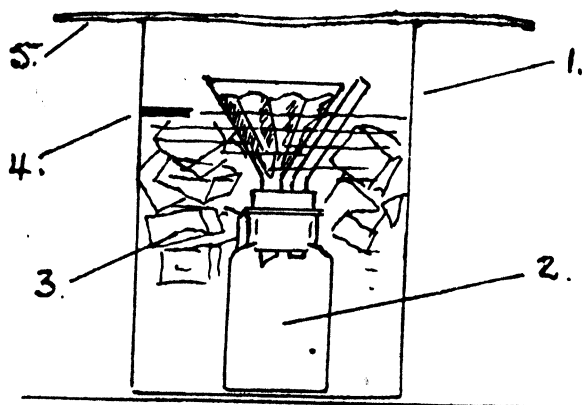


FIG. 1. Cold filtration in hot climates.

1. 1,000 ml. beaker.
2. Filtration unit with air-vent, resting upon the base of the beaker; placed in position 10 minutes before use. In practice there is no danger of the filtration unit upsetting.
3. Mixture of ice, salt and water at 0°–1° C. Serum and reagents mixed in a corked test-tube are kept in this medium for 30 minutes before filtering.
4. Water-level marked on the beaker, ensuring that the depth of water is always 1 cm. less than the height of the filtration unit.
5. Cardboard cover minimizing air-movement within the beaker.

2. Determination of the Nitrogen Content of Whole Serum and Globulin-Free Filtrate

The method available to the present author for the determination of the nitrogen content of whole serum and globulin-free filtrate involved micro-Kjeldahl digestion followed by nesslerization and colorimetry. Selenium catalysts were not available. The technique was substantially similar to that quoted by Hoch and Marrack (1945) in their review of the comparative yields of various digestion procedures—a technique which was found by them to yield 96.5 per cent. recovery.

Comparison of the values found in 20 of the sera in the present series with the values given by the copper sulphate specific gravity method (Phillips *et al.*, 1944–45) (adopting the formula of Hoch and Marrack) indicated a 93 per cent. recovery; if the original formula

of Phillips *et al.* was applied, the recovery stood at 96·8 per cent. The figures have been adjusted on the assumption that the recovery was 95 per cent.—a procedure which may have resulted in a 2 per cent. understatement of the total protein and albumin figures.

Non-protein nitrogen was taken as 25 mgm. per cent., in view of the fact that African blood-urea levels do not differ materially from European normals.

Certain precautions in regard to colorimetry were observed. Ammonium sulphate standards were so arranged that the 'unknown' did not differ from the appropriate standard by more than 11 per cent. In these conditions the reagent-blank (itself equivalent to 7 per cent. of the average appropriate standard) could not affect the final readings by more than 0·8 per cent.

All estimations were made in triplicate (as also were the digestions), and the colorimeter reading-error was reduced to some 2 per cent. by averaging nine successive readings. All estimations were performed by the writer.

RESULTS

The figures obtained are summarized in the following table.

TABLE

	Albumin	Globulin	Total	Albumin/globulin ratio
AFRICANS (gm. per cent.)				
Range	2·1-4·4	2·9-4·8	6·0-8·2	0·5-1·4
Mean	3·45	3·76	7·21	0·93
Standard deviation ...	0·47	0·45	0·55	0·21
Standard error of mean ...	0·086	0·081	0·099	0·039
EUROPEANS (gm. per cent.)				
Range	3·5-4·9	1·8-3·3	5·8-7·8	1·2-2·7
Mean	4·3	2·56	6·86	1·7
Standard deviation ...	0·31	0·35	0·403	0·296
Standard error of mean ...	0·056	0·064	0·073	0·054

No correlation was apparent in the case of Europeans between albumin/globulin ratio and period of residence in tropical Africa.

The observed differences between the African and the European means in respect of albumin, globulin and albumin/globulin ratio are certainly statistically significant; the ratio between these differences and their respective standard errors being 8+, 11+, and 11+.

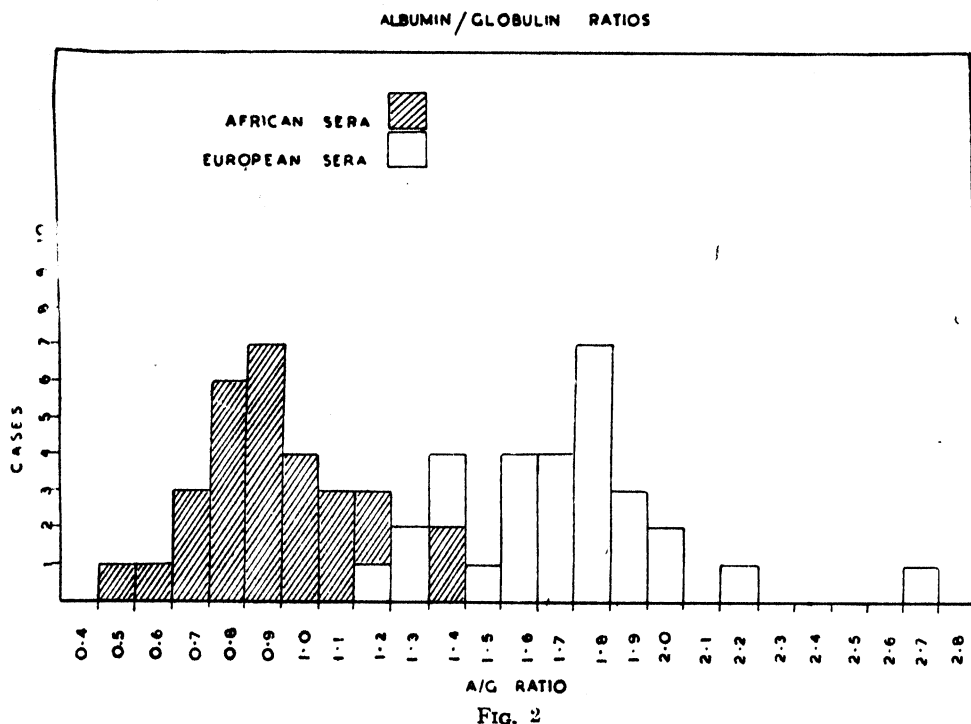
The observed difference between the means in respect of total protein is 2·8 times its standard error, which theoretically indicates that the chances of its being significant lie between 80 to 1 and 370 to 1. In view of the comparatively small number of cases involved, it would be unwise to assume, without further extension of the series, that the difference is in fact significant.

The frequency distribution of the albumin/globulin ratios is shown in fig. 2; it will be seen that the values tend to a normal distribution.

DISCUSSION

Although the series is small in number, it seems to be established that these African albumin/globulin ratios are different from European, and that this difference is due to a shift in the levels of both albumin and globulin rather than to an absolute deficiency of one fraction.

Sera from five pregnant African women gave albumin/globulin ratios varying from 0.45 to 0.7, total proteins ranging from 6.0 to 6.8 gm. per cent. Sera from five African children between the ages of three and eight years, all considered to be suffering from nutritional deficiency, gave albumin/globulin ratios ranging between 0.3 and 0.9, total proteins lying between 5.5 and 8.0 gm. per cent. The child giving the lowest of these



figures showed clinical oedema; albumin amounted to 1.2 gm. per cent., globulin to 4.3 gm. per cent. That the globulin may be greatly raised, in circumstances which suggest that the rise is compensatory to albumin loss, is shown by a case of hepatic cirrhosis in a young adult African, whose serum proteins amounted to 6.6 gm. per cent., to which globulin contributed 6.0 gm. per cent. and albumin only 0.6 gm. per cent.—an albumin/globulin ratio of 0.1.

More detailed fractionation of the serum proteins in Africans by the techniques developed in America, references to which are given by Cohn (1945), might throw light on the reasons for the increased globulin levels. Africans are subject to considerable antigenic barrage in childhood and appear to be markedly susceptible to liver disease; diet is an obvious factor.

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TAXONOMY OF THE ETHIOPIAN SANDFLIES (*PHLEBOTOMUS*)

I.—CLASSIFICATION AND SYNONYMY

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INTRODUCTION

Several years ago, one of us, being interested in kala-azar, suggested to the entomological staff in the Sudan* that a sandfly-survey of that country would be desirable. It was pointed out to us that what we wanted was no small matter, since the taxonomy of *Phlebotomus* was a highly specialized subject which very few persons were competent to undertake. Consultations with other entomological authorities confirmed this view, and the best handbook for the identification of insects of medical importance known to us, published in 1943, dismisses the genus with the remark that 'the specific determination of species of *Phlebotomus* is a matter for the specialist.'

Difficulty in the identification of *Phlebotomus* arises from the small size of the insects and from the fact that in most cases specific characters are found only in the internal structures, so that each specimen has to be cleared, dissected under the binocular, and then examined under low and high powers of the microscope before it can be identified. In our experience, however, the greatest difficulty of all arises from the scattered nature of the literature, which is, nevertheless, copious. Sinton (1932, 1933) published illustrated keys for the identification of males and females, which form a useful starting-point as regards the Indian species; but no such guide is available for the Ethiopian species. In our studies of the sandflies of the Sudan we found that the only way to avoid confusion was to copy from the various journals descriptions and figures of each species, and to bind these abstracts together in a single file. By so doing we were able to collect in convenient form an up-to-date account of all the species which had been found in the Sudan and adjacent countries. From this it was possible to construct keys and diagnostic tables, which proved of great assistance in the identification of specimens.

It has been suggested that these keys might be useful to other workers, as there is evidence of a growing interest in this important genus of biting insects; so the present article is presented in the hope that others may find it at least a useful guide to the copious and scattered literature on this subject. Lists of African sandflies, with appropriate references, have been published by Sinton (1930), Theodor (1931a) and De Meillon and Lavoipierre (1944); but, with the exception of Theodor's paper, which includes a key to the species, these are merely catalogues and do not enter into the questions of classification and identification. Theodor's paper dealt with some 20 species, including the Mediterranean as well as the purely African fauna, and the number of species recorded from Africa has increased greatly since that paper was published.

* In this paper Sudan means the Anglo-Egyptian Sudan.

CLASSIFICATION

The following discussion on the classification of *Phlebotomus* is concerned only with the species occurring in the Old World, and does not include the American species.

Newstead (1911) divided the sandflies of the Maltese Islands into two groups : (i) those in which the hairs on the dorsum of the abdomen are erect, and (ii) those in which these hairs are recumbent. A similar division was used by Sinton (1924) in compiling a diagnostic table of the males of the Indian species.

França (1919) made an attempt to classify the species of *Phlebotomus* by dividing them into two subgenera on the characters of the male genitalia. Later, França and Parrot (1921) divided the genus into five subgenera, namely, *Prophlebotomus*, *Phlebotomus* s. restr., *Brumptomyia*, *Lutzia* (emend. *Lutzomyia* França, 1924), and *Sergentomyia*. In this classification *Prophlebotomus* was separated from the other subgenera by the alar index being less than unity in *Prophlebotomus* and greater than unity in the other subgenera. The remaining subgenera were separated according to differences in the male genitalia. This classification was unsatisfactory in many ways. In a later publication, Parrot (1934b) points out that the scheme was conceived as a guide to further work and research rather than as a categorical affirmation of definitive characters.

In 1926 Adler and Theodor showed from an examination of Palestine sandflies that in certain species the posterior portion of the buccal cavity bears a number of teeth and a pigmented area of chitin, which are absent in other species. They showed, further, that the morphology of these structures and of the pharynx and spermathecae were of diagnostic value in the determination of species. In 1927 Sinton found that Newstead's division into erect- and recumbent-haired species was correlated with definite morphological characters in the spermathecae of the females, species of the erect-haired group having spermathecae with a crenulated or segmented appearance, while in the recumbent-haired group this organ has a smooth outline. The discovery of these new characters greatly facilitated the specific determination of *Phlebotomus*. Sinton (1927) suggested a division of the genus into three main divisions, which were further subdivided as follows :

A. *The erect-haired division*

The members of this division have always some erect hairs on the dorsal aspects of segments II to VI of the abdomen. The heavily chitinized parts of the bases of the spermathecae of the females of this division are segmented in their entire length. The species in this division may be arranged into two groups :

Group 1. The erect abdominal hairs are numerous, usually occurring in tufts at the distal ends of the segments ; dorsal recumbent hairs very scanty or absent on abdominal segments II to VI ; buccal armature and pigmented area absent or rudimentary ; the spermathecal chitinizations and male hypopygium usually having specific characters, and the pharyngeal armature showing a useful diagnostic morphology.

Group 2. Species with scanty erect hairs on the dorsal aspects of abdominal segments II to VI ; such hairs often confined to segments II and III, especially in the males ; dorsal recumbent hairs usually numerous ; buccal armature and pigmented area usually well developed and with specific morphology.

B. *The recumbent-haired division*

In this division the dorsal abdominal hairs on segments II to VI are all recumbent; the body of the spermathecal chitinizations usually with a smooth outline, and any traces of segmentation, if present, are confined to the distal end. The buccal armature and pigmented area are usually well developed and have a specific morphology. The division contains two groups:

Group 3. In these the morphology of the male genitalia closely resembles that of *P. minutus* Rondani.

Group 4. The morphology of the male genitalia in this group is distinctly different from that of *P. minutus* Rondani.

C. *The intermediate division*

Only one species, *P. squamipleuris*, is contained in this division. This species may show a few erect hairs on the dorsal aspects of some of the abdominal segments from II to VI, but more usually all the hairs are recumbent in this position. The spermathecal chitinizations are not crenulated, but show a series of transverse rows of small spines. This species differs from all others in that the thoracic pleurae carry tufts of broad scales like those seen in mosquitoes, in the unilateral geniculate spines on the antennae of the female, in the absence of those spines on segment III, and in the presence of Newstead's sensory spines on both the second and third palpal segments.

Other workers who have attempted the classification of *Phlebotomus* (Nitzulescu, 1931; Theodor, 1931b) appear to regard the buccal cavity as of greater diagnostic value than the disposition of the hairs on the abdominal tergites, and divide the genus into two primary divisions—species which possess a buccal armature and those without a buccal armature. Nitzulescu found that, in spite of its apparent simplicity, Sinton's primary division into erect- and recumbent-haired species was not always easy to determine in practice, and proposed a division of the genus into five subgenera, as follows:

A. *Possessing a well-marked buccal armature*

1. Spermatheca segmented.....Subgenus *Sintonius*
2. Spermatheca not segmented.....Subgenus *Brumptius*

B. *Buccal cavity without teeth and pigmented area*

3. Spermatheca segmented, with long neck.....Subgenus *Larrousius*
4. Spermatheca segmented, with short neck.....Subgenus *Phlebotomus*
5. Spermatheca smooth ('lisse').....Subgenus *Adlerius*

Parrot (1934a) takes exception to this classification on the grounds that it places closely related species like *P. major* and *P. chinensis* in different subgenera, whereas such diverse forms as *P. papatasi*, *P. argentipes* and *P. sergenti* are grouped together in one subgenus, and also because the classification depends entirely on the characters of the female and does not take into account the male genitalia, the importance of which was recognized by previous workers. Parrot agrees, however, with the primary division into species which possess a well-developed buccal armature and those which do not, but states his opinion that it is hardly justifiable to undertake any further subdivision of the genus at

the present time, at least as far as the Old World species are concerned. He points out that the first group corresponds in large measure with the subgenus *Prophlebotomus* of França and Parrot (1921), and divides the genus into two subgenera, which are defined as follows :

'*Phlebotomus* : bouche inerme ; indice alaire supérieur ou au moins égal à l'unité ; poils des tergites abdominaux uniformément dressés ;—parasites habituels des animaux à sang chaud. Type : *P. papatasi* (Scop., 1786).

'*Prophlebotomus* França et Parrot, 1921 : bouche armée de dents ; indice alaire généralement inférieur ou à peine égal à l'unité ; poils des tergites abdominaux uniformément ou presque uniformément couchés ;—parasites habituels des animaux à sang froid. Type : *P. minutus* Rond., 1843.'

Sinton (1928) found wing-venation a variable character unsuited for primary division, and several species of the subgenus *Prophlebotomus* have since been recorded as vicious biters of man (cf. Kirk and Lewis, 1940). The primary division into those forms which have a buccal armature and those which do not is, however, a convenient one. It was found very useful by us in our studies of the sandflies of the Sudan because it enables any single male or female, the precise determination of which is doubtful, to be assigned to the appropriate group.

It will be seen that the subgenus *Phlebotomus* corresponds with Sinton's group 1. Groups 2, 3, 4 and the intermediate division of Sinton are all included in the subgenus *Prophlebotomus*. In the course of fairly extensive studies on the genus *Phlebotomus* we have come to recognize Sinton's group 2 as a fairly homogeneous group, presenting specific features, which, if the morphology of the spermatheca and hairs on the abdominal tergites be taken into consideration, are rather intermediate between *Phlebotomus* and the other members of the subgenus *Prophlebotomus*. The females correspond with Nitzulescu's definition of the subgenus *Sintonius*, and there is considerable uniformity among the males of this group, all those which have been described so far having genitalia of the *minutus* type, with hooked intermediate appendage and sharp intromittent organ (fig. 17*). We therefore think it convenient to separate this group from the subgenus *Prophlebotomus* of França and Parrot (1921), and to recognize three subgenera of *Phlebotomus* in Africa as follows :

Phlebotomus s. restr. Species with numerous erect hairs on the abdominal tergites, usually occurring in tufts on the distal ends of the segments ; buccal armature and pigmented area absent or rudimentary ; the heavily chitinized parts of the spermathecae segmented in their entire length ; spermathecal chitinizations and male hypopygium usually having specific characters.

Sintonius Nitzulescu, 1931. Species with scanty erect hairs on the dorsal aspects of abdominal segments II to VI, such hairs often confined to segments II and III, especially in the males ; dorsal recumbent hairs usually numerous ; buccal armature and pigmented area usually well developed and showing a specific morphology ; spermathecae crenulated ; male hypopygium usually of the *minutus* type, with pointed intromittent organ and hooked intermediate appendage.

Prophlebotomus França and Parrot, 1921. Species having dorsal abdominal hairs on

* The figures referred to here and below will appear in Part II of this paper, which will be published later.

segments II to VI, all recumbent; buccal armature and pigmented area usually well developed and having a specific morphology; the body of the spermathecal chitinizations usually with a smooth outline, and any traces of annulation, if present, confined to the distal end.

Of these three subgenera, *Sintonius* is the most homogeneous. The easily recognizable characters of the subgenus are, however, found only in the females. In species which are known only by the male it may be difficult to differentiate between *Sintonius* and *Prophlebotomus* if the genitalia are of the *minutus* type and if the dorsal abdominal hairs have been displaced, as is usually the case in preserved and mounted specimens. Sinton (1932) has pointed out, however, that in specimens which have been mounted, especially if stained, it is comparatively easy to determine the original disposition of the hairs from a study of the scars left on the abdominal segments. The scars left by erect hairs are much larger and more refractile than those left by recumbent hairs (fig. 1).

The subgenus *Prophlebotomus* comprises a variety of different forms, including species like *P. squamipleuris* and *P. wurtzi*, in which the spermathecae are peculiar. The subgenus *Phlebotomus* includes *P. gigas*, which has non-crenulated spermathecae (Parrot and Schwetz, 1937). In both these subgenera there is considerable variety in the structure of the genitalia in the males.

SYNONYMY OF *P. MINUTUS*

Compared with many other groups, the taxonomy of *Phlebotomus* is not greatly encumbered by synonymy. The main difficulty is concerned with the synonymy of *P. minutus*. *P. minutus* is not an African species, but a brief review of its synonymy is necessary in an account of African sandflies.

The genus *Phlebotomus* was established by Rondani in 1840, though the species for which it was founded had been placed by various authorities in other genera, such as *Bibio* (Scopoli, 1786), *Musca* (Gmelin, 1788-93), *Cinipes* (Costa, 1840). *P. minutus* was originally described by Rondani in 1843 from Italy, and Loew (1844) redescribed *P. papatasi* Scopoli under the same name in the following year. The first full account of *P. minutus* in modern literature is that of Newstead (1911) from Malta. Later Newstead (1912) separated *P. minutus* var. *africanus* from the type on account of certain differences in external characters.

In 1926 Adler and Theodor drew attention to the value of the buccal and pharyngeal armatures and of the spermatheca in the taxonomy of *Phlebotomus*. In two Palestinian forms determined as *P. minutus* and *P. minutus* var. *africanus* they found that the differences in external characters were correlated with differences in the internal structure. *P. africanus* was accordingly raised to specific rank. A collection of *Phlebotomus*, determined on external characters as *P. minutus* and *P. africanus*, was found to contain *P. africanus* and two other species, one similar to the *P. minutus* of Palestine, the other a species which could not be differentiated from *P. minutus* on external characters, but showed very clear differences from *P. minutus* in the buccal cavity and pharynx. It was therefore described as *P. parroti* Adler and Theodor, 1927.

The discovery of the taxonomic importance of the buccal and pharyngeal armatures and of the spermatheca stimulated studies on *Phlebotomus*. In the following years many new species and varieties were described. *P. minutus* was recorded from many localities

in Asia and Africa. Sinton (1928) examined one of Newstead's co-type specimens of *P. minutus* from Malta and found that it agreed with the Palestinian form. Later, however, Sinton (1933) states in a footnote that 'The species now commonly known as *P. minutus* is that described by Adler and Theodor (1926, 1927) from Palestine. It is doubtful whether this is the same as *P. minutus* Rondani, 1843, from Italy. The latter may be synonymous with *P. parroti* Adler and Theodor, 1927.'

This apparently is so. The matter has recently been cleared up by Parrot (1942, 1943), who found that the only *Phlebotomus* occurring in Malta and Italy was, in fact, a variety of *P. parroti*. This, he concluded, must therefore be the original *P. minutus* Rondani, and he redescribed it under this name in 1943. The Palestinian form originally described as *P. minutus* by Adler and Theodor was renamed *P. theodori*, and other alterations in nomenclature were made to clarify the position of various forms which had previously been assimilated as varieties of *P. minutus*.

The nomenclature used by us in this paper is that which results from Parrot's recent clarification of the synonymy of *P. minutus* Rondani, 1843. *P. minutus* is essentially a Palaearctic species and does not occur in Africa. Previous records of this species from Africa are therefore invalid, as they refer to some other species, most commonly *P. signatipennis* Newstead.

ETHIOPIAN SPECIES OF PHLEBOTOMUS

In the following catalogue of the Ethiopian species of *Phlebotomus* we have given with each species references to publications in which systematic descriptions and illustrations of the species will be found. The signs ♂ or ♀ attached to a reference indicate that a description or figure of the species necessary for its identification will be found in the paper referred to. We have given also such additional references as are necessary to trace the synonymy of species which have been reported under different names. In the case of varieties we have noted also the points of difference between the variety and the type, so as to eliminate the necessity for including the varieties in the diagnostic tables which follow.

SUBGENUS PHLEBOTOMUS

P. langeroni var. *orientalis* Parrot.

P. langeroni var. *orientalis*, Parrot (1936), *Arch. Inst. Pasteur Algér.*, **14**, 30 (♂ ♀).

P. perniciosus, Archibald and Mansour (1937), *Trans. Roy. Soc. Trop. Med. & Hyg.*, **30**, 395.

P. perniciosus, Sinton (1937), *Ibid.*, **30**, 404.

P. perniciosus, Kirk (1939), *Ibid.*, **32**, 541.

P. langeroni, Theodor (1938), *Bull. Ent. Res.*, **29**, 165.

P. langeroni var. *orientalis*, Kirk and Lewis (1940), *Trans. Roy. Soc. Trop. Med. & Hyg.*, **33**, 623.

P. langeroni was separated as a variety of *P. perniciosus* by Nitzulescu (1930), on the characters of a non-bifid intromittent organ and a different antennal formula, and was later raised to specific rank by the same author. In 1936 Parrot described, from Abyssinia, *P. langeroni* var. *orientalis*, in which the intromittent organ is non-bifid but the antennal formula is similar to that of *P. perniciosus*.

The specimen described by Theodor (1938) from the Sudan as *P. langeroni* was probably var. *orientalis*, but as the antennae were missing this could not be decided.

The records of *P. perniciosus* in the Sudan, reported by Archibald and Mansour (1937), Sinton (1937) and Kirk (1939), all refer to one specimen, a male collected by one

of us (D. J. L.) in the Blue Nile area and sent to England for identification in 1936. Professor Adler suggested in a letter that there might be some confusion in this record between *P. perniciosus* and *P. langeroni* var. *orientalis*. We were able to trace the specimen and to verify (1940) that the provisional determination of *P. perniciosus* was later changed to *P. langeroni* var. *orientalis*. *P. perniciosus* should not, therefore, be included among the Ethiopian species of *Phlebotomus*.

Distribution. Abyssinia, Sudan, Northern Kenya.

P. longipes Parrot and Martin.

P. longipes, Parrot and Martin (1939), *Arch. Inst. Pasteur Algér.*, 17, 143 (♂ ♀).

Distribution. Abyssinia, Sudan.

P. sergenti Parrot.

P. sergenti, Parrot (1917), *Bull. Soc. Path. Exot.*, 10, 564 (♂).

P. sergenti, França (1918), *Ibid.*, 11, 731 (♀ ♂).

P. sergenti, Adler and Theodor (1929), *Ann. Trop. Med. & Parasitol.*, 23, 271 (♀).

Distribution. French West Africa, Central Sahara.

P. sergenti var. *saevus* Parrot and Martin.

P. sergenti var. *saevus*, Parrot and Martin (1939), *Arch. Inst. Pasteur Algér.*, 17, 484.

The male differs from the type in the arrangement of the tuft of hairs on the proximal segment of the superior clasper, and in the shape of the peduncle bearing these hairs. One of the two distal spines on the distal segment of the superior clasper is markedly sub-terminal, whereas in the type both spines are terminal. In the female the size is greater and the pharyngeal teeth are finer and more numerous than in the type.

Distribution. Abyssinia, Sudan.

P. alexandri Sinton.

P. sergenti var., Newstead (1920), *Bull. Ent. Res.*, 11, 305 (♂).

P. sergenti var. *alexandri*, Sinton (1928), *Ind. J. Med. Res.*, 16, 297.

P. alexandri, Parrot (1936), *Arch. Inst. Pasteur Algér.*, 14, 428 (♀).

Newstead (1920) described from Mesopotamia, but did not name, a variety of *P. sergenti* which differed from the type in having the third antennal segment much shorter and the distal bifurcated spine-bearing processes of the superior clasper markedly unequal in length, the terminal process being about three times the length of the other. Sinton (1928) later recorded this form from India and proposed the name var. *alexandri*, as its recorded distribution was similar to the eastern conquests of Alexander the Great. It was later recorded from Greece and parts of North Africa by Parrot, who in 1936 raised the variety to specific rank as *P. alexandri* Sinton.

Distribution. Southern Sudan.

P. martini Parrot.

P. martini, Parrot (1936), *Arch. Inst. Pasteur Algér.*, 14, 30 (♂ ♀).

Distribution. Abyssinia, Sudan.

P. rossi De Meillon and Lavoipierre.*P. rossi*, De Meillon and Lavoipierre (1944), *Jl. Ent. Soc. S. Afr.*, **7**, 44 (♂).*Distribution.* Southern Rhodesia.*P. katangensis* Bequaert and Walravens.*P. katangensis*, Bequaert and Walravens (1930), *Rev. Zool. Bot. Afr.*, **19**, 35 (♂).*Distribution.* Belgian Congo.*P. papatasii* Scopoli.*P. papatasii*, Newstead (1911), *Ann. Trop. Med. & Parasitol.*, **5**, 139 (♂ ♀).*P. papatasii*, Sinton (1928), *Ind. Jl. Med. Res.*, **15**, 300.*P. papatasii*, Sinton and Barraud (1928), *Ibid.*, **16**, 325 (♀).

This insect has been described so frequently and is now so well known that any account of its synonymy is principally of historical interest. The papers of Newstead and Sinton quoted above give a full account of the early synonymy of the species, which it is unnecessary to repeat here.

Distribution. Sudan, Somaliland, French West Africa. Also widely distributed in the Mediterranean area and in western Asia.

P. papatasii var. *bergeroti* Parrot.*P. papatasii* var. *bergeroti*, Parrot (1934), *Arch. Inst. Pasteur Algér.*, **12**, 383 (♂).*P. papatasii* var. *bergeroti*, Parrot (1936), *Ibid.*, **14**, 39.*P. viduus*, Parrot (1936), *Ibid.*, **14**, 34 (♀).*P. papatasii* var. *bergeroti*, Parrot (1941), *Ibid.*, **19**, 437 (♀).

The variety *bergeroti* was originally separated from the type by Parrot (1934) from specimens of males from the Central Sahara, on account of the greater relative length of the third antennal segment, the smaller tuft of hairs on the proximal segment of the superior clasper, and the position of the submedian spines on the distal segment (fig. 15). In 1936 Parrot recorded this species from Abyssinia, together with the female of a new species, *P. viduus*. Further observations showed a close association between *P. viduus* ♀♀ and *P. papatasii* var. *bergeroti* ♂♂, leading to the conclusion that the former was, in fact, the female of *P. papatasii* var. *bergeroti*, and that *P. viduus* was a *nomen nudum* (Parrot, 1941). The female is distinguished from the type by the greater length of the geniculate spines on the antennae, the more heavily armed pharynx, and the spermatheca, consisting of 5–8 segments only.

Distribution. Abyssinia, Central Sahara, Sudan.

P. duboscqi Neveu-Lemaire.*P. duboscqi*, Neveu-Lemaire (1906), *Bull. Soc. Zool. France*, **31**, 65 (♂).*P. duboscqi*, Picard (1909), *Bull. Soc. Ent. France*, 164.

This species was created by Neveu-Lemaire in 1906, but the description of it is deficient in essential characters. Picard (1909), who re-examined the type-specimen, states that he was unable to distinguish any difference between it and *P. papatasii*. The status of the species is uncertain at present and re-examination of the type-specimens is required to determine its relation to *P. roubaudi*, *P. papatasii* and *P. papatasii* var. *bergeroti* (see below).

Distribution. French West Africa.

P. roubaudi Newstead.

P. roubaudi, Newstead (1913), *Bull. Soc. Path. Exot.*, **6**, 124 (♂).

P. duboscqi, França and Parrot (1921), *Arch. Inst. Pasteur Afr. N.*, **1**, 283.

P. roubaudi, Parrot and Gougis (1944), *Ibid.*, **22**, 40 (♂ ♀).

This species was described by Newstead (1913) from specimens collected in the region in which the type-specimens of *P. duboscqi* were obtained. Although showing specific characters, *P. roubaudi* resembles *P. papatasi* closely, and Newstead pointed out that it may be identical with *P. duboscqi*, the species created but incompletely described by Neveu-Lemaire in 1906. Detailed descriptions of both sexes of *P. roubaudi* were published in 1944 by Parrot and Gougis, who consider that as the name *P. roubaudi* refers to an adequately described form it should be regarded as correct unless the matter can be finally settled by re-examination of the type-specimens of *P. duboscqi*.

Distribution. French West Africa, Sudan, Nigeria, Ashanti, Mauretania, Sierra Leone.

P. rodhaini Parrot.

P. rodhaini, Parrot (1930), *Rev. Zool. Bot. Afr.*, **19**, 181; *Ibid.*, **20**, 103 (♂).

Distribution. Belgian Congo, Sudan.

P. gigas Parrot and Schwetz.

P. gigas, Parrot and Schwetz (1937), *Rev. Zool. Bot. Afr.*, **29**, 224 (♀).

P. gigas, Parrot and Wanson (1938), *Ibid.*, **31**, 153 (♂).

Distribution. Belgian Congo.

SUBGENUS SINTONIUS

P. adleri Theodor.

P. adleri, Theodor (1933), *Bull. Ent. Res.*, **24**, 543 (♂ ♀).

Distribution. Gold Coast, Nigeria, Sudan.

P. affinis Theodor.

P. affinis, Theodor (1933), *Bull. Ent. Res.*, **24**, 545 (♀).

P. affinis, Lewis and Kirk (1940), *Proc. Roy. Ent. Soc. Lond. (B)*, **9**, 127 (♂).

Distribution. Sudan.

P. caffaricus De Meillon and Lavoipierre.

P. caffaricus, De Meillon and Lavoipierre (1944), *Jl. Ent. Soc. S. Afr.*, **7**, 46 (♀).

Distribution. Cape Province.

P. clydei Sinton.

P. clydei, Sinton (1928), *Ind. Jl. Med. Res.*, **16**, 179 (♂ ♀).

P. clydei, Lewis and Kirk (1939), *Proc. Roy. Ent. Soc. Lond. (B)*, **8**, 155 (♂ ♀).

P. vagus, Parrot and Martin (1939), *Arch. Inst. Pasteur Algér.*, **17**, 147.

P. clydei, Parrot and Martin (1944), *Ibid.*, **22**, 55 (♂ ♀).

P. clydei was originally described by Sinton (1928) from specimens from Waziristan, and has since been found to have a wide distribution in the plains of India. In 1939 Lewis and Kirk recorded this species from the Sudan, where its distribution was also extensive. These authors described certain minor differences between the type and the African

specimens, and pointed out the resemblance between the latter and *P. vagus*, which Parrot and Martin had recently described from Abyssinian specimens. Parrot and Martin (1944), in a later paper, concluded that *P. vagus* was a synonym of *P. clydei*.

Distribution. Sudan, Abyssinia, Djibuti, French West Africa. Also in India.

P. matadiensis Theodor.

P. matadiensis, Theodor (1938), *Bull. Ent. Res.*, 20, 165 (♂ ♀).

Parrot (1939) has drawn attention to the close similarity between this species and *P. wansoni* Parrot 1938. Both species were described from specimens from the same locality. *P. wansoni* is distinguished by the presence of spines on the femora, which are not mentioned in the description of *P. matadiensis*.

Distribution. Belgian Congo.

P. meilloni Sinton.

P. meilloni, Sinton (1932), *Ind. Jl. Med. Res.*, 20, 565 (♂ ♀).

Distribution. Transvaal, Southern Rhodesia, Swaziland.

P. meilloni var. *suberectus* Sinton.

P. meilloni var. *suberectus*, Sinton (1932), *Ind. Jl. Med. Res.*, 20, 565 (♂).

This variety differs from the type in a number of relative measurements, but the most marked difference is in the arrangement of the buccal teeth, which number 20–25 instead of 10.

Distribution. Kenya.

P. subtilis Parrot and Martin.

P. tiberiadis, Parrot (1936) (nec Adler, Theodor and Lourie, 1930), *Arch. Inst. Pasteur Algér.*, 15, 44 (♀).

P. subtilis, Parrot and Martin (1939), *Ibid.*, 17, 151 (♂).

P. subtilis, Parrot and Martin (1940), *Ibid.*, 18, 300 (♂ ♀).

Parrot (1936) reported provisionally as *P. tiberiadis* Adler and Theodor a female from Abyssinia. This form was later found in association with males which Parrot and Martin had described in 1939 as *P. subtilis*. Further studies showed that the form provisionally reported as *P. tiberiadis* was in fact the female of *P. subtilis*, and a full description of both sexes was published in 1940 by Parrot and Martin.

Distribution. Abyssinia.

P. thomsoni Theodor.

P. thomsoni, Theodor (1938), *Bull. Ent. Res.*, 29, 167 (♂ ♀).

Distribution. Nyasaland.

P. transvaalensis Sinton.

P. transvaalensis, Sinton (1933), *Ind. Jl. Med. Res.*, 20, 879 (♀).

Distribution. Transvaal.

P. wansoni Parrot.*P. wansoni*, Parrot (1938), *Rev. Zool. Bot. Afr.*, 30, 361 (♂).*P. wansoni*, Parrot (1939), *Ibid.*, 32, 147 (♀).*Distribution.* Belgian Congo, French West Africa.

SUBGENUS PROPHLEBOTOMUS

P. africanus Newstead.*P. minutus* var. *africanus*, Newstead (1912), *Bull. Ent. Res.*, 3, 363 (♂ ♀).*P. africanus*, Adler and Theodor (1926), *Ibid.*, 16, 402.*P. africanus*, Sinton (1928), *Ind. J. Med. Res.*, 16, 297.*P. africanus*, Adler, Theodor and Parrot (1929), *Rev. Zool. Bot. Afr.*, 18, 73 (♂ ♀).*P. africanus*, Theodor (1933), *Bull. Ent. Res.*, 24, 541 (♀).

P. minutus var. *africanus* was separated from the type *P. minutus* Rondani by Newstead (1912) on account of its darker colour and certain differences in external morphology. In 1926 Adler and Theodor showed in their classical paper that the buccal and pharyngeal armatures of the variety were also distinct from the type, and raised the variety to specific rank.

Probably several species have been recorded as *P. africanus* in the past. Many varieties of this species have been created since 1926. There are still some curious discrepancies in the literature referring to the type. Adler, Theodor and Parrot (1929) depict *P. africanus* from the Belgian Congo with no anterior small teeth in the buccal cavity. Theodor (1933) states that the form which occurs in the Sudan and Congo is typical, and depicts the buccal armature of the female of this form with anterior punctiform teeth. Newstead's original specimen came from Rhodesia. Sinton (1928) examined one of Newstead's paratypes from Southern Nigeria and concluded that it was identical with the Palestinian form described by Adler and Theodor. But so many varieties of this species have now been recorded from Africa that the original type-specimen from North-Eastern Rhodesia requires re-examination.

Distribution. *P. africanus* and its varieties are widely distributed in Africa. Also in western Asia.

P. africanus var. *ater* Parrot.*P. africanus* var. *ater*, Parrot (1936), *Arch. Inst. Pasteur Algér.*, 14, 43 (♀).

Buccal teeth 46–48, pigmented area in the form of a crescent strongly curved with convexity anterior, not occupying whole width of buccal cavity. No anterior teeth in buccal armature. Palpal formula 1, 2, (3, 4), 5.

Distribution. Abyssinia.*P. africanus* var. *longior* Parrot.*P. africanus* var. *longior*, Parrot (1936), *Arch. Inst. Pasteur Algér.*, 14, 40 (♂ ♀).

Buccal teeth 48–50 in female, and pigmented area as in the type but with six anterior punctiform teeth. The male has 20–25 buccal teeth (as compared with 15–20 in the type) and a relatively long intromittent organ.

Distribution. Abyssinia.

P. africanus var. *magnus* Sinton.

P. africanus var. *magnus*, Sinton (1932), *Ind. Jl. Med. Res.*, **20**, 571 (♀).

This variety was described from one female from South Africa and was given varietal rank because it differed in some measurements from *P. africanus* of Palestine, resembling more closely that of the Congo (Adler, Theodor and Parrot, 1929). Theodor (1933) believes it to be merely a large specimen of *P. africanus*.

Distribution. Transvaal.

P. africanus var. *meridianus* De Meillon and Lavoipierre.

P. africanus var. *meridianus*, De Meillon and Lavoipierre (1944), *Jl. Ent. Soc. S. Afr.*, **7**, 44 (♀).

The female has only 20 buccal teeth and palpal formula 1, 2, (3, 4), 5.

Distribution. Transvaal.

P. africanus var. *niger* Parrot and Schwetz.

P. africanus var. *niger*, Parrot and Schwetz (1937), *Rev. Zool. Bot. Afr.*, **29**, 226 (♂ ♀).

The female has 60–65 teeth in the buccal armature with no anterior small teeth. The pigmented area is extremely dark. The male has 30–36 buccal teeth.

Distribution. French Congo, Belgian Congo, Nigeria, Sudan, French West Africa.

P. africanus var. *sudanicus* Theodor.

P. africanus var. *sudanicus*, Theodor (1933), *Bull. Ent. Res.*, **24**, 541 (♀).

Female has 30–33 buccal teeth with an anterior row of small teeth, arranged one at the base of every second tooth among the armature.

Distribution. Sudan, French West Africa.

P. freetownensis Sinton.

P. freetownensis, Sinton (1930), *Ind. Jl. Med. Res.*, **18**, 188 (♀).

Distribution. Sierra Leone.

P. babu Annandale.

P. babu, Annandale (1910), *Rec. Ind. Mus.*, **4**, 49 (♂ ♀).

P. babu, Sinton (1928), *Ind. Jl. Med. Res.*, **16**, 314.

P. babu, Sinton (1932), *Ibid.*, **20**, 55 (♀); (1933), *Ibid.*, **21**, 417 (♂).

Distribution. Mauritius. Also widespread in parts of Asia.

P. bedfordi Newstead.

P. bedfordi, Newstead (1914), *Bull. Ent. Res.*, **5**, 191 (♀).

P. bedfordi, Parrot (1921), *Arch. Inst. Pasteur. Afr. N.*, **1**, 269 (♂).

Descriptions of this species, which resembles *P. africanus*, have been made on external morphology only, and its status will have to be determined by re-examination of the type-specimens.

Distribution. Transvaal.

P. yvonnae Parrot and Schwetz.

P. yvonnae, Parrot and Schwetz (1937), *Rev. Zool. Bot. Afr.*, **29**, 221 (♀).

Distribution. Belgian Congo.

P. collarti Adler, Theodor and Parrot.

P. collarti, Adler, Theodor and Parrot (1929), *Rev. Zool. Bot. Afr.*, **18**, 81 (♂ ♀).

Distribution. Belgian Congo.

P. decipiens Theodor.

P. simillimus, Adler, Theodor and Parrot (1929) (nec Newstead, 1914), *Rev. Zool. Bot. Afr.*, **18**, 72 (♂ ♀).

P. decipiens, Theodor (1931), *Bull. Ent. Res.*, **22**, 473 (♂ ♀).

Adler, Theodor and Parrot described as *P. simillimus* a species from the Congo which agreed in its external characters with Newstead's description of that species. Re-examination of Newstead's type-specimens showed later that the two species were distinct, and the one from the Congo was redescribed as *P. decipiens*.

Distribution. Belgian Congo, Sudan.

P. simillimus Newstead.

P. simillimus, Newstead (1914), *Bull. Ent. Res.*, **5**, 180 (♂ ♀).

P. brodeni, Parrot (1930), *Rev. Zool. Bot. Afr.*, **19**, 185 (♀).

P. simillimus, Theodor (1931), *Bull. Ent. Res.*, **22**, 475 (♂ ♀).

P. simillimus, Theodor (1938), *Ibid.*, **29**, 171 (♂).

P. simillimus was described in 1914 by Newstead from specimens from West Africa. In 1929 Adler, Theodor and Parrot obtained a species from the Belgian Congo which in its external features agreed with Newstead's description of *P. simillimus*; they therefore described the internal morphology of the species under the same name. Comparison with Newstead's co-type specimens later showed, however, that the two species were distinct, and Theodor (1931) redescribed *P. simillimus* from Newstead's co-type specimens, giving further data in 1938. In the meantime Parrot (1930) described from the Congo, under the name of *P. brodeni*, a species which was different from that previously described by Adler, Theodor and Parrot as *P. simillimus*. As this species agrees with Theodor's description of co-types of *P. simillimus*, *P. brodeni* becomes a synonym of that species.

Distribution. Ashanti, Gold Coast, Belgian Congo, Sudan.

P. signatipennis Newstead.

P. signatipennis, Newstead (1920), *Bull. Ent. Res.*, **11**, 305 (♀).

P. minutus, Theodor (1931), *Ibid.*, **22**, 469.

P. minutus var. *antennatus*, Parrot (1930), *Rev. Zool. Bot. Afr.*, **19**, 189.

P. sanneri, Galliard and Nitzulescu (1931), *Ann. Parasitol. Hum. Comp.*, **9**, 233.

P. minutus var. *signatipennis*, Theodor (1933), *Bull. Ent. Res.*, **24**, 537 (♀).

P. signatipennis, Parrot (1942), *Arch. Inst. Pasteur Algér.*, **20**, 322 (♂ ♀).

The species *P. signatipennis* was created by Newstead (1920) on the character of a very small alar index ($\alpha/\beta = 0.2$). After examination of several specimens determined by Newstead as *P. signatipennis*, Theodor (1931) concluded that there was no real difference between these and *P. minutus*, but in a later paper (Theodor, 1933) depicts the buccal armature of *P. minutus* var. *signatipennis* as differing from other varieties of *P. minutus* in the greater length and width of the median teeth. With the redescription of *P. minutus* Rondani by Parrot (1942) it becomes evident that the form *signatipennis* can no longer be regarded as a variety of *P. minutus*. So *P. signatipennis* was again raised to specific rank by Parrot, who points out that *P. sanneri*, described by Galliard and Nitzulescu (1931)

from the Gabon and recorded by Parrot from Abyssinia, should be regarded as a synonym of *P. signatipennis*.

Distribution. Sudan, Gabon, Abyssinia, Belgian Congo, French West Africa ; also occurs in North Africa.

P. cinctus Parrot and Martin.

P. cinctus, Parrot and Martin (1944), *Arch. Inst. Pasteur Algér.*, **22**, 57 (♀).

Distribution. Djibuti.

P. antennatus Newstead.

P. antennatus, Newstead (1912), *Bull. Ent. Res.*, **3**, 365.

P. minutus var. *antennatus*, Newstead and Sinton (1921), *Ann. Trop. Med. & Parasitol.*, **15**, 103.

P. antennatus, Newstead (1920), *Bull. Ent. Res.*, **11**, 305.

P. antennatus, Sinton (1933), *Ind. J. Med. Res.*, **21**, 417.

P. minutus var. *antennatus*, Theodor (1933), *Bull. Ent. Res.*, **24**, 539.

P. antennatus, Parrot (1942), *Arch. Inst. Pasteur Algér.*, **20**, 322.

P. antennatus was originally described by Newstead as a distinct species (1912, 1920), but later Newstead and Sinton (1921) thought the evidence then available was insufficient to separate it from *P. minutus*, and placed it as a variety of that species. Sinton (1933) later found that the differences between both male and female forms of Indian specimens of the two insects were so distinct that he considered that *P. antennatus* should again be raised to specific rank, although the identity of the Indian form of the species with the original African type was uncertain. Theodor (1933) found two specimens among a collection of *P. occidentalis* from the Gold Coast presenting many characters similar to those figured by Sinton for the Indian form of *P. antennatus*, but with a different palpal formula. Minor differences in the buccal teeth and the relative lengths of the third antennal segment were also found between the Indian and African forms, and Theodor concluded that the two forms were not identical. It is possible, as Sinton suspected, that the Indian form will have to be renamed, but the nomenclature cannot be settled without a re-examination of Newstead's African specimens.

With Parrot's redescription of *P. minutus* it is evident that the various forms regarded as *antennatus* are not varieties of *P. minutus*. The form described by Parrot (1930) from the Congo as *P. minutus* var. *antennatus* is now regarded by that worker as *P. signatipennis* (Parrot, 1942).

Distribution. Gold Coast.

P. dubius Parrot, Mornet and Cadenat.

P. minutus var. *antennatus*, Theodor (1933), *Bull. Ent. Res.*, **24**, 539.

P. dubius, Parrot Mornet and Cadenat (1945), *Arch. Inst. Pasteur Algér.*, **23**, 232 (♂ ♀).

Under the name *P. dubius* Parrot, Mornet and Cadenat have recently described the form reported from the Gold Coast by Theodor (1933) as *P. minutus* var. ?*antennatus* Newstead. They point out that this form is certainly different from the *P. antennatus* described from India by Sinton, but its relation to *P. antennatus* Newstead can only be settled by re-examination of Newstead's type-specimens, which may be impossible. In the circumstances they consider it desirable to give this form another specific name, which will stand unless the matter can be finally settled by re-examination of the type-specimens of *P. antennatus* Newstead.

Distribution. Gold Coast, French West Africa.

P. occidentalis Theodor.

P. minutus var. *occidentalis*, Theodor (1933), *Bull. Ent. Res.*, 24, 542 (♀).

P. occidentalis, Parrot (1942), *Arch. Inst. Pasteur Algér.*, 20, 332.

Theodor noted a fairly distinctive appearance in the buccal armature of specimens of *P. minutus* from the West Coast of Africa, and gave those specimens varietal rank. With Parrot's redescription of *P. minutus* it is evident that the West African form can no longer be regarded as a variety of that species, and Parrot suggests that it should be raised to specific rank as *P. occidentalis*.

Distribution. Gold Coast, Nigeria.

P. buxtoni Theodor.

P. buxtoni, Theodor (1933), *Bull. Ent. Res.*, 24, 544 (♂ ♀).

P. mathisi, Parrot (1935), *Arch. Inst. Pasteur Algér.*, 13, 259 (♂ ♀).

Distribution. Gold Coast, Senegal.

P. congolensis Bequaert and Walravens.

P. africanus var. *congolensis*, Bequaert and Walravens (1930), *Rev. Zool. Bot. Afr.*, 19, 38 (♂ ♀).

P. nairobiensis, Theodor (1931), *Bull. Ent. Res.*, 22, 472 (♂ ♀).

P. congolensis, Parrot (1933), *Rev. Zool. Bot. Afr.*, 23, 239.

P. congolensis, Theodor (1933), *Bull. Ent. Res.*, 24, 542.

Bequaert and Walravens (1930) separated *P. africanus* var. *congolensis* from the type on account of the form of the intromittent organ (pointed in *africanus*, blunt in *congolensis*). Both sexes were redescribed by Parrot (1933), who raised the variety to specific rank as *P. congolensis* and pointed out that the characters of this species were identical with those described by Theodor for *P. nairobiensis*. After examination of specimens of *P. congolensis*, Theodor (1933) agreed that the two were identical.

Distribution. Abyssinia, Sudan, East Africa, Belgian Congo, Southern Rhodesia, Transvaal.

P. congolensis var. *distinctus* Theodor.

P. congolensis var. *distinctus*, Theodor (1933), *Bull. Ent. Res.*, 24, 542 (♂ ♀).

The female of this variety differs from the type in having 24 instead of 34-40 teeth in the buccal cavity. The difference in size between the median and lateral teeth is more marked, and the pharynx is broader and more heavily armed. In the male Aiii/E is greater than unity, whereas in the type this ratio is less than unity.

Distribution. Sudan, Gold Coast, Nigeria, French West Africa.

P. congolensis var. *firmatus* Parrot and Malbrant.

P. congolensis var. *firmatus*, Parrot and Malbrant (1945), *Arch. Inst. Pasteur Algér.*, 23, 122 (♂ ♀).

This variety differs from the type in a number of minor characters, particularly in the greater relative length of the antennal geniculate spines in both sexes, in the smaller number of buccal teeth in the female (16-20), and in the ratio Aiii/E in the male, which is greater than unity instead of less than unity, as in the type.

Distribution. French Congo.

P. yusafi Sinton.*P. yusafi*, Sinton (1930), *Ind. Jl. Med. Res.*, **18**, 181 (♂ ♀).*Distribution.* Kenya.*P. schoutedeni* Adler, Theodor and Parrot.*P. schoutedeni*, Adler, Theodor and Parrot (1929), *Rev. Zool. Bot. Afr.*, **18**, 79 (♂ ♀).*Distribution.* Belgian Congo, Uganda, Sudan, Transvaal.*P. ingrami* Newstead.*P. ingrami*, Newstead (1914), *Bull. Ent. Res.*, **5**, 180 (♂).*P. ingrami*, Adler, Theodor and Parrot (1929), *Rev. Zool. Bot. Afr.*, **18**, 87 (♂ ♀).*P. ingrami*, Theodor (1933), *Bull. Ent. Res.* **24**, 546.*Distribution.* Ashanti, Belgian Congo, Ivory Coast, Uganda, Sudan, Gold Coast.*P. serratus* Parrot and Malbrant.*P. serratus*, Parrot and Malbrant (1945), *Arch. Inst. Pasteur Algér.*, **23**, 125 (♀).*Distribution.* French Congo, Sudan.*P. renauxi* Parrot and Schwetz.*P. renauxi*, Parrot and Schwetz (1937), *Rev. Zool. Bot. Afr.*, **29**, 222 (♀).*Distribution.* Belgian Congo.*P. schwetzi* Adler, Theodor and Parrot.*P. schwetzi*, Adler, Theodor and Parrot (1929), *Rev. Zool. Bot. Afr.*, **18**, 75 (♂ ♀).*P. symesi*, Sinton (1930), *Ind. Jl. Med. Res.*, **18**, 175 (♂ ♀).*P. schwetzi*, Theodor (1931), *Bull. Ent. Res.*, **22**, 471.*Distribution.* Sudan, Transvaal, Kenya, Gold Coast, French Congo, Belgian Congo, Nigeria, French West Africa.*P. schwetzi* var. *aethiopicus* Parrot.*P. schwetzi* var. *aethiopicus*, Parrot (1936), *Arch. Inst. Pasteur Algér.*, **14**, 39 (♂ ♀).Var. *aethiopicus* differs from the type principally in its greater length, in the longer and straighter intromittent organ in the male, and in the narrower pharynx in the female.*Distribution.* Abyssinia, Sudan.*P. notatus* Parrot.*P. notatus*, Parrot (1938), *Arch. Inst. Pasteur Algér.*, **16**, 216 (♀).*Distribution.* Abyssinia.*P. durenii* Parrot.*P. durenii*, Parrot (1934), *Rev. Zool. Bot. Afr.*, **24**, 266 (♀).*P. durenii*, Parrot (1939), *Ibid.*, **32**, 145 (♂).*Distribution.* Belgian Congo.

P. wurtzi Parrot.

P. wurtzi, Parrot (1938), *Arch. Inst. Pasteur Algér.*, **16**, 213 (♂ ♀).

Distribution. Abyssinia.

P. mirabilis Parrot and Wanson.

P. mirabilis, Parrot and Wanson (1939), *Rev. Zool. Bot. Afr.*, **32**, 149 (♂ ♀).

Distribution. Belgian Congo.

P. squamipleuris Newstead.

P. squamipleuris, Newstead (1912), *Bull. Ent. Res.*, **3**, 366 (♀).

P. squamipleuris, Sinton (1923), *Ind. J. Med. Res.*, **11**, 65; (1927), *Ibid.*, **15**, 24 (♂ ♀).

P. ghesquieri, Parrot (1929), *Rev. Zool. Bot. Afr.*, **18**, 90.

P. squamipleuris, Parrot (1930), *Ibid.*, **19**, 182 (♂ ♀).

P. squamipleuris, Theodor (1931), *Bull. Ent. Res.*, **22**, 470.

Newstead described this species from a single female from Khartoum. In 1923 Sinton recorded the species from India and described both sexes, adding the description of the characteristic buccal armature and spermatheca in 1927. *P. ghesquieri*, described by Parrot from the Congo, was later shown by the same author to be a synonym of *P. squamipleuris*. Theodor in 1931 pointed out certain differences between the African and the Indian forms of this species, and separated the latter from the type as *P. squamipleuris* var. *indicus*.

Distribution. Abyssinia, Madagascar, Mozambique, Nigeria, Sudan, French West Africa. Also India and other parts of Asia.

P. squamipleuris var. *dreyfussi* Parrot.

P. squamipleuris var. *dreyfussi*, Parrot (1933), *Arch. Inst. Pasteur Algér.*, **11**, 603 (♀).

The female differs from the type in having very large spermathecae, 44 instead of 36–40 buccal teeth, and spines on the femora.

Distribution. Abyssinia; also North Africa.

P. squamipleuris var. *inermis* Theodor.

P. squamipleuris var. *inermis*, Theodor (1938), *Bull. Ent. Res.*, **29**, 165 (♀).

The female differs from the type in having 20–24 buccal teeth only and poorly developed pharyngeal armature.

Distribution. Abyssinia, Nigeria.

P. viator Parrot and Martin.

P. viator, Parrot and Martin (1939), *Arch. Inst. Pasteur Algér.*, **17**, 143 (♂).

Following Parrot and Martin (1939) we have included this species provisionally in the subgenus *Prophlebotomus*. The species is known by the male only, and it is possible that the discovery of the female will show it to be one of the subgenus *Sintonius*.

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THE USE OF AVIAN MALARIA FOR THE DISCOVERY OF DRUGS EFFECTIVE IN THE TREATMENT AND PREVENTION OF HUMAN MALARIA

I.—DRUGS FOR CLINICAL TREATMENT AND CLINICAL PROPHYLAXIS

BY

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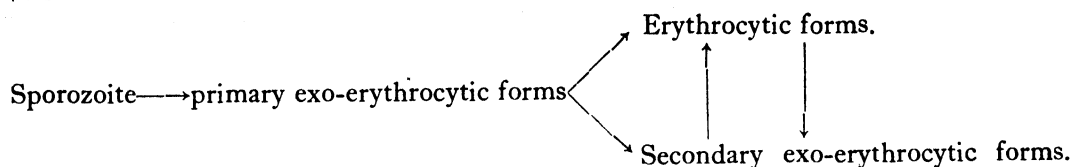
(Received for publication November 17th, 1945)

In a previous paper (Curd, Davey and Rose, 1945a) a method was described by which an infection of *Plasmodium gallinaceum* in chicks was used in the discovery of drugs efficacious for the relief of symptoms in human malaria. The description was written more than two years ago, but, because some of the matter came under the Official Secrets Act, its publication was temporarily suspended. During those two years much more work has been done, and other methods of testing substances for their antimalarial action, ancillary to the main method, have been developed and have proved their value. It is the purpose of this paper to describe the reasons why the different methods were evolved and the uses to which they are put. The substances which are used to illustrate the experimental results were prepared under the direction of Dr. F. H. S. Curd and Dr. F. L. Rose.

There are several hundreds of references in the literature to the effect of some particular drug on one or more of the various species of *Plasmodium*—they are quoted in the reviews by Bishop (1942), Curd (1943) and Marshall (1942)—but for the most part they deal with passing observations or with studies of special points, such as the mode of action of a drug, and are not concerned with the paramount interest, experienced by us during the war, of using avian malaria simply as an instrument for the fashioning of new antimalarial substances. Roehl experienced a similar interest when he developed his canary test (Roehl, 1926), and many workers in the United States of America have shared it during the war. Much of the American work done under the auspices of the Board for the Co-ordination of Malarial Studies has been made known to us through our collaboration with the Medical Research Council, and ours has been made known to them. We followed the Americans in making our inoculation of parasites intravenously, but for the rest our tests have been designed to suit our own requirements. The variations between us, however, have usually been a matter of detail—detail of technique or detail of interpretation—although sometimes differences of approach are apparent. The latter has been largely governed by man-power. In this laboratory one trained biologist with a group of technical assistants has been responsible for the organization and maintenance of all the testing; in the U.S.A. it appears that half-a-dozen or so fully equipped teams have specialized in particular aspects of it.

The main infection on which all the work in this laboratory has been based has continued to be *P. gallinaceum* in chicks. The probable life-cycle of this parasite was outlined

in a letter to *Nature* (Davey, 1944) and was depicted (with a slight change of terminology) as follows :



Huff and Coulston (1944), following some most painstaking and beautifully executed work, have described in detail for the first time the complete succession of stages between the sporozoites and the parasites of the red blood-cells, and have thus demonstrated conclusively that, at least in *P. gallinaceum*, a period of development of the parasite occurs in the solid tissues before the blood-cells are invaded. Their observations on the life-cycle, together with ours, will be discussed in the second paper of this series, and it will be sufficient at present to point out that the only forms of the malarial parasite which have been observed in human malaria after the inoculation of sporozoites are those of the red blood-corpuses, and that it is the multiplication of these parasites which brings about clinical symptoms. The first part of the malarial problem, the control of clinical symptoms, is therefore the chemotherapy of the erythrocytic forms, and is what concerns us now. The second part, the complete prevention of a type of malaria such as benign tertian by the daily administration of drugs, or its complete cure, might be associated with the chemotherapy of exo-erythrocytic forms and will be discussed later.

THE TEST AGAINST *P. GALLINACEUM* IN CHICKS

The main points in the test using *P. gallinaceum* in chicks are briefly as follows :

(a) Chicks, six days old and weighing 45–55 gm., are injected intravenously with approximately 50 million parasitized cells. The injection is conveniently done on Monday. The inoculum is prepared from the pooled blood of chicks infected four days previously by the intravenous injection of about 100 million parasitized cells. Clotting of the pooled blood is prevented by heparin. A cell count is made of it and also, from a stained smear, the proportion of cells which are parasitized is determined. The two results give the approximate number of parasitized cells in the pooled blood, which is then diluted with Ringer's solution to give the required 50 million parasitized cells in each 0.2 ml. of inoculum.

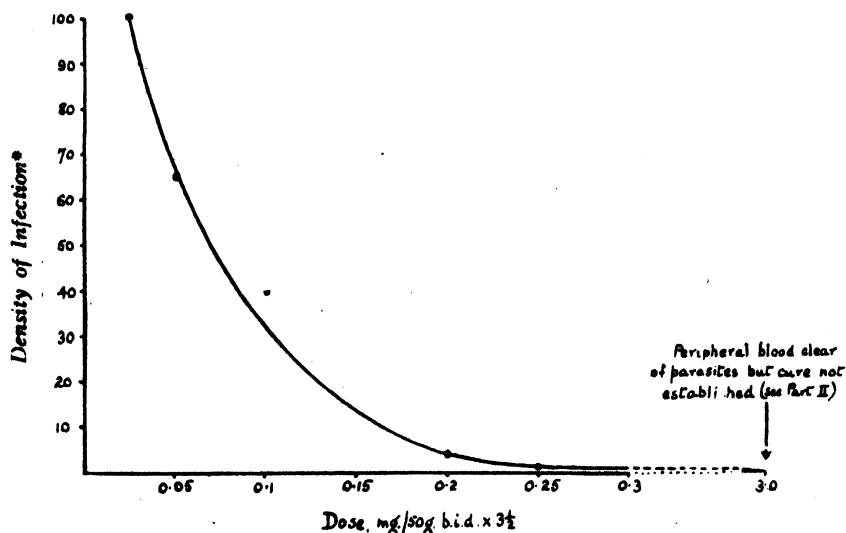
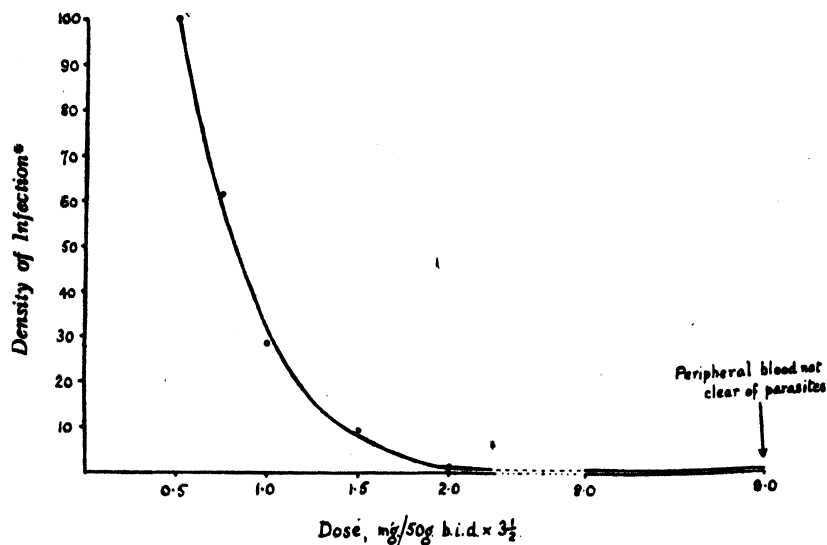
(b) The treatment of the chicks with the test substances commences about four hours after they have been infected, and is repeated twice daily, at 9–9.30 a.m. and 4.30–5 p.m., on each of the next three days (Tuesday, Wednesday and Thursday). The substances are given orally, in solution or suspension, through a catheter-tube passed into the gizzard. The strength of the solution is such that a dose for a chick weighing 50 gm. is contained in 1 ml. Usually six chicks are included for each dose of each drug and for the control group.

(c) Blood smears, made from the base of the leg-vein, are taken on the morning of the fifth day (Friday) about 18 hours after the last dose was given. The smears are stained in the usual way with Giemsa.

(d) The density of the infection is expressed as the number of parasitized corpuscles in a random sample of 500.

(e) The activity of a drug is assessed in the first place by searching for what can be

described as its critical dose region. The upper limit of this region is approximately the lowest dose of the substance exerting what is materially its maximum effect; the lower limit is approximately the lowest dose exerting a measurable effect. For our purposes



* The density of infection is expressed as a percentage of the density in untreated chicks.

we regard the upper limit as being the minimum effective dose of the substance (M.E.D.), and we use it to make an approximate comparison of the activity of the substances tested. These definitions will be best understood from a consideration of a concrete example.

If the response to treatment of an infection with *P. gallinaceum* is plotted against a series of progressively increasing doses of drug, a curve is obtained similar to those illustrated in the accompanying figure, which shows (a) the response to mepacrine and (b) the response to Paludrine acetate (4888). There is first a period during which little or no effect is being recorded. Then the threshold of measurable activity is reached, and each subsequent small augmentation in the size of the dose, over a comparatively short range, yields a marked effect until the response is almost full. Afterwards, increasing the dose many times yields comparatively little result. A full effect, or in other words a complete cure, is not obtained with mepacrine because it is without the requisite action on exo-erythrocytic forms, but even using a drug such as Paludrine, with which a complete cure is theoretically possible, a similar curve is obtained.

The critical dose region, as defined above, clearly delimits that part of the curve where the slope is most steep, i.e., where the response to treatment is very markedly influenced by a slight change in the size of the dose. In the case of mepacrine it is 1-2 mgm., measured in terms of the individual dose given to a 50 gm. chick twice daily for 3½ days, and for Paludrine it is 0.1-0.25 mgm. As we define it, therefore, the M.E.D. for mepacrine is 2 mgm. and for Paludrine is 0.25 mgm.

No attempt has been made to specify the exact magnitude of the effect which is achieved by the M.E.D., but it can be said to be characterized by the *uniformly* low parasite counts obtained in the treated birds. There are, of course, undoubtedly fewer parasites in a chick treated with 8 mgm. of mepacrine than in one treated with 2 mgm., but nevertheless what is *materially* the maximum effect is achieved by 2 mgm. At 2 mgm. the count is low, and there is uniformity amongst all the treated birds; below 2 mgm., on the steep part of the curve, uniformity in the results in different birds is lost, and while some readings may be low others may approach the level seen in untreated birds. Lack of uniformity in the results obtained in a group of treated birds is, in fact, a clear indication of slight activity on the part of the substance.

How accurately the M.E.D. is fixed depends entirely on one's requirements. In this laboratory it is usual for the doses to be graded in a geometric series with a ratio of two. For example, a series of doses may be 4 mgm., 2 mgm., 1 mgm., 0.5 mgm., and two of them may fix in an approximate way the critical dose region. Thus, as can be seen from Table I, it is 1-2 mgm. for mepacrine, quinine and 3349. (Note that in this table and all subsequent ones, unless otherwise stated, doses are given in mgm./50 gm., and were administered b.i.d. $\times 3\frac{1}{2}$. The density of each infection is expressed as the number of parasitized corpuscles per 500 examined; in the ratio x/y , x is the arithmetical average for birds of the treated group, y for the controls.)

So many factors enter into the final consideration of a substance for clinical trial—these are discussed below—that we have not considered it necessary to delimit the activity of these substances any more accurately than by the doses given. For mepacrine, quinine and 3349, then, the M.E.D. is approximately 2 mgm., and they are therefore about equally active. The results at 1 mgm. show, however, that if the assay of their activity were done more delicately mepacrine would probably be somewhat better than 3349. This can be seen from the detailed results given in Table II, which were obtained at this dose in several tests with the two substances; these results also show clearly the variation in response associated with a treatment which is only slightly effective.

TABLE I

Activity of mepacrine, quinine and 3349 in the oral test against *P. gallinaceum*. The results are the averages of at least two tests

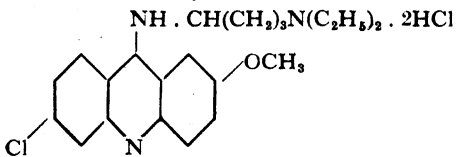
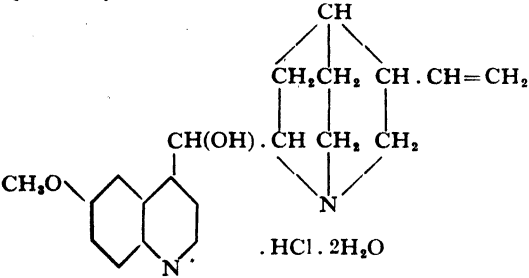
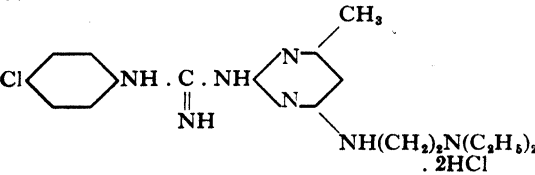
Drug	Results at				
	4 mgm.	2 mgm.	1 mgm.	0.5 mgm.	M.E.D.
Mepacrine hydrochloride CH_3 $\text{NH} \cdot \text{CH}(\text{CH}_2)_3\text{N}(\text{C}_2\text{H}_5)_2 \cdot 2\text{HCl}$ 	1/366	3/359	129/359	309/359	2 mgm.
Quinine hydrochloride  $\cdot \text{HCl} \cdot 2\text{H}_2\text{O}$	1/315	7/398	188/367	368/367	2 mgm.
3349  $\cdot 2\text{HCl}$	3/314	10/314	217/373	381/373	2 mgm.

TABLE II

Results in individual chicks after the administration of 1 mgm.
b.i.d. of mepacrine and 3349

Drug	Individual results	Average
Mepacrine	14, 22, 26, 32, 36, 67, 68, 68, 98, 157, 170, 176, 198, 208, 240, 248, 264, 372	136.8
• 3349	45, 124, 176, 204, 241, 244, 248, 256, 258, 280, 288, 332, 332, 345, 348, 350, 364, 368	266.8
Controls	264, 300, 307, 315, 324, 325, 331, 366, 375, 375, 391, 396, 403, 406, 412, 421, 432, 456	366.6

An M.E.D. which is quoted in this paper, then, is approximately the lowest dose of a drug which, given twice daily to a 50 gm. chick for $3\frac{1}{2}$ days, achieves what is materially the maximum effect of the drug against the blood forms short of actual sterilization; its value is approximate within the limits set out above. Periodically, the M.E.D. of mepacrine has been redetermined as a check on the testing methods, and it has been gratifying to find how constant the results have been. This fact is demonstrated by Table III, which gives the results obtained with mepacrine at the two doses of 2 mgm. and 1 mgm. at different times. It will be seen that, although the figures for 1 mgm. vary somewhat, there is never any doubt that with this dosage-point the steep part of the response curve has been reached, and that 2 mgm. invariably achieves what is virtually the full effect.

TABLE III
Results obtained with mepacrine in the oral test against
P. gallinaceum at different times

Date	2 mgm.	1 mgm.
July 26th, 1943 ...	4.8/314	207/314
September 6th, 1943 ...	3/363	131/363
November 22nd, 1943 ...	3.5/327	76.5/327
February 21st, 1944 ...	5.5/422	172/422
September 11th, 1944 ...	2/357	214/357
" 21st, 1945 ...	3/417	116.5/417

Constancy in results is only brought about by a rigid standardization of the test. The chicks used in these experiments have been bought always from the same hatchery. They have been crosses between Rhode Island Red, Light Sussex and Black Sussex, and mostly only the cocks have been used, but no difference in susceptibility between the sexes or the various crosses has been detected in six-day-old chicks, which was the age-group used for all the work. A selection of the birds has always been made, and only those weighing between 45 and 55 gm. have been put on experiment. They have always been inoculated intravenously with approximately 50 million parasitized red cells.

Standardization within these limits causes the peak of the parasitaemia to be reached in the majority of the birds on the fifth day of the test, which is the day on which smears are made; but a variation in either the age of the chicks or the size of the inoculum will

TABLE IV
Variations in the response of *P. gallinaceum* in chicks to
mepacrine according to the size of inoculum

Approximate no. of parasitized red cells in inoculum	Percentage reduction in the density of infection at the specified dose measured on the 5th day	
	2 mgm.	1 mgm.
250 million	83%	50%
100 "	94%	61%
50 "	99.25%	73%
10 "	99.4%	92%

alter the time at which the peak is reached, and either bias or favour the results obtained with a drug. This is shown in Table IV, in which some results are quoted which were obtained with mepacrine using inocula of different sizes. Similar results were obtained with quinine.

An inoculum of approximately 50 million parasitized cells was chosen for our experiments because it is about the lowest which allows the peak of the parasitaemia to be reached in the majority of birds on the fifth day of the test and in other ways is generally convenient. With inocula higher than 100 million a slightly active drug may be overtaxed, and with inocula lower than about 25 million the variation amongst the controls is rather great. For example, in an experiment in which the inoculum consisted of 10 million parasitized erythrocytes the density of the infection on Friday in the control chicks, expressed as usual as the number of parasitized cells found during the examination of a random sample of 500 corpuscles, varied between 65 and 232, and on Saturday between 176 and 475. Inocula of 50 million parasitized erythrocytes, on the other hand, rarely give variations in the controls greater than 100 on the fifth day, and usually the counts of the parasitized corpuscles lie between 300 and 400. It has been pointed out that, in this test, much variation between the results in the individuals in a treated group is indicative of a slight effect by the drug. The variation, of course, will be reflected in the average for the group, and statistical treatment of the results* has shown that, when the average for the control readings are at the usual level of 300 to 400, a difference of about 100 in the average readings for a treated group is indicative of a chemotherapeutic effect.

The critical dose region of a drug is influenced not only by the size of the inoculum, but obviously, too, by the frequency of the dosing. If this is once daily the region for mepacrine is 2-4 mgm., and if only one dose is given, about four hours after infection, it lies between 6 and 8 mgm.

THE SELECTION OF A SUBSTANCE FOR CLINICAL TRIAL

In Table V are given the results obtained with some of the more active substances discovered during our work and with some of the standard antimalarial drugs. The table is divided into two parts; listed in the first part are substances the activity of which is approximately the same as that of mepacrine and quinine, and in the second part others the activity of which is greater and may in some instances approach that of pamaquin, the most active substance in avian malaria yet discovered.

On the face of it, all the substances listed in Table V are worth a trial in human malaria, and it is hoped that all of them will eventually be given one, but at the time when they were discovered such a course was not possible and a further selection had to be made. This was again a consequence of the restrictions which the war had placed upon us. Admittedly, it is not difficult to make the requisite studies for a substance to receive clinical trial, but they involve much labour. A chemist must make about 100 gm. of it; detailed toxicological studies and pharmacological studies must be done with it; the data must be assessed, and the conclusions then given to the clinician in charge of the trial in human malaria. Our clinical trials have been conducted in the first place at the Liverpool School of Tropical Medicine, with whose staff an intimate collaboration has

* I am indebted to Dr. O. L. Davies for this information.

TABLE V (a)
Activity of substances in the oral test against *P. gallinaceum*

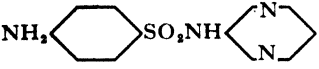
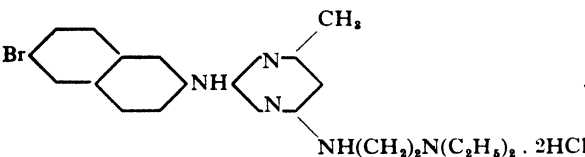
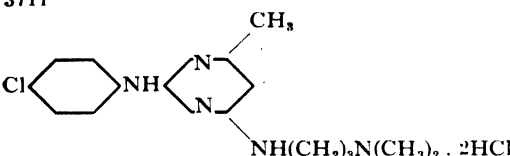
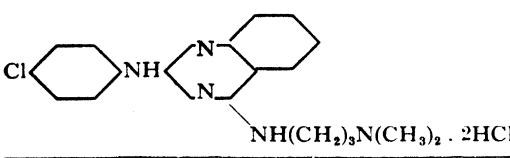
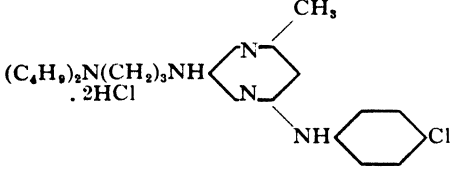
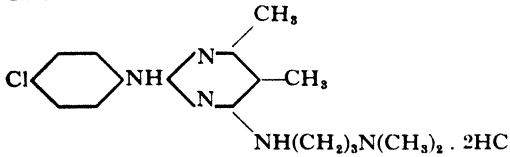
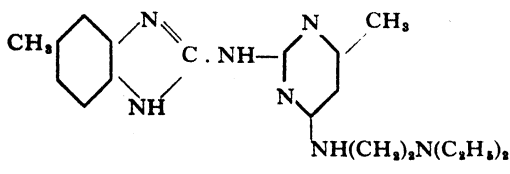
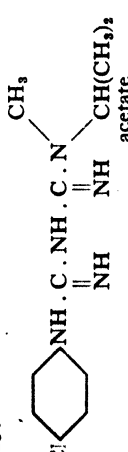
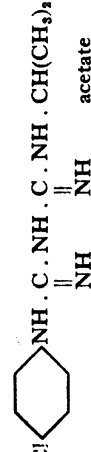
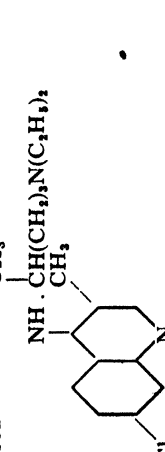
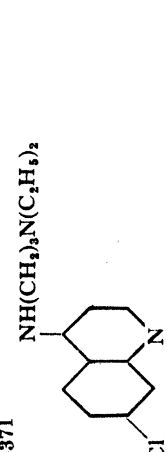
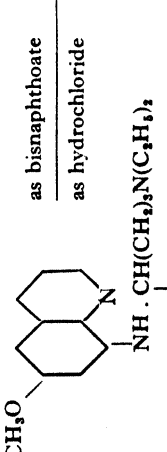
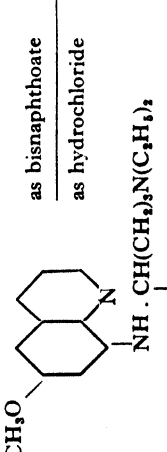
Substance	Results at			M.E.D.
	4 mgm.	2 mgm.	1 mgm.	
Sulphadiazine				
		40/364	142/364	2-3 mgm.
3502				
	4/366	13/363	111/363	2 mgm.
3711				
	1/373	9/441	44/441	2 mgm.
3979				
		17/379	189/379	2 mgm.
4316				
	3/396	4/372	227/372	2 mgm.
4410				
		6/410	67/410	2 mgm.
4821				
		21/380	131/380	2 mgm.

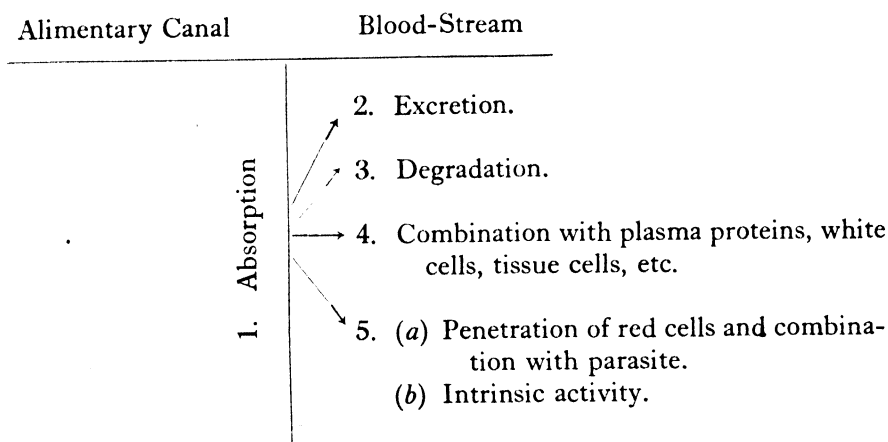
TABLE V (b)

Substance	Results at							M.E.D.
	2 mgm.	1 mgm.	0.5 mgm.	0.25 mgm.	0.1 mgm.	0.05 mgm.	0.025mgm.	0.01 mgm.
4430  <chem>CC1(C)C(=O)N(C1C2=CC=CC=C2Cl)C3=CC=CC=C3N</chem>	2/320	4/365	84/365	225/365				1 mgm.
Paludrine acetate								
 <chem>CC(=O)N1C2=CC=CC=C2N1C3=CC=CC=C3Cl</chem>	1/332	2/332	2/400	1/398	250/398			0.25 mgm.
4592  <chem>CC1(C)C(=O)N(C1C2=CC=CC=C2Cl)C3=CC=CC=C3N</chem>	1/356	3/356	144/356					1 mgm.
5371  <chem>CC1(C)C(=O)N(C1C2=CC=CC=C2Cl)C3=CC=CC=C3N</chem>		0.6/251	3/369	15.1/407	233/320	333/320		0.25-0.5 mgm.
Pamaquin  <chem>CC1(C)C(=O)N(C1C2=CC=CC=C2Cl)C3=CC=CC=C3N</chem>				1/327	4/329	266/315		0.1 mgm.
as bisnaphthoate as hydrochloride  <chem>CC1(C)C(=O)N(C1C2=CC=CC=C2Cl)C3=CC=CC=C3N</chem>					2/338	5/338	182/357	0.05 mgm.

been maintained. Their resources were often considerably strained in helping the work along, and it was clear to all of us that their difficulties, coupled with ours, prevented every active compound being given a clinical trial immediately. If our study had been done at leisure we might, perhaps, have allowed each active substance to wait its turn; but it happened that events were forcing us to work as speedily as possible, and consequently we were compelled to determine by further experiments in the laboratory any differences between our active substances which might have a bearing on their activity in human malaria.

The necessity for doing so became apparent early in the investigations. In the paper which describes in detail the test with *P. gallinaceum* (Curd, Davey and Rose, 1945a) the considerations governing the choice of a substance for clinical trial were discussed. In the main, it was argued that the choice depended on the activity of the substance when tested orally against *P. gallinaceum* and its toxicity for a mammal; if the results of these tests, compared with the results similarly obtained for mepacrine, appeared to justify the step, the substance was selected for trial. This was the approach which led to the selection of 3349, a substance which was efficacious in controlling the clinical attack of all types of human malaria when given in doses of 200 mgm. three times daily. Shortly afterwards 3502 (the constitutions and activities of these compounds are given in Tables I and V) was discovered, and in some ways its chances of success against human malaria appeared more favourable than those of 3349. Amongst other things it was active against *P. lophurae*, while 3349 was inactive (see below); but, in any event, when it was tried in cases of benign tertian and malignant tertian malaria it failed to achieve a clinical effect at doses as high as 200 mgm. given three times a day for seven days. Clearly, explanations were called for.

The various factors which may influence the activity of a drug can be listed diagrammatically as follows:



Following the administration of a particular quantity of drug, the amount entering the blood-stream will depend, of course, on the rate of absorption. It would be difficult to define an optimum rate, but at least it should be sufficiently fast to allow absorption to be reasonably complete. Once in the blood-stream the drug will become exposed to the

forces of excretion and degradation, and some of it, too, might be bound, loosely or firmly, on a variety of tissue elements. Ideally one would desire the quantity coming under the influence of these forces to be as small as possible, since, from the viewpoint of a therapeutic effect, it probably represents drug which is wasted. Some of the drug, however, will penetrate the red cells and come in contact with the parasite, and clearly the net effect of the treatment will depend on the amount which does this and on the intrinsic antimalarial activity of the drug. In other words, the outcome of what may be described as the competition between host and parasite for the drug is, in its way, as important in determining the final result as the intrinsic activity of the drug. We can presume that the competition is a dynamic one and that the amount of drug being distributed between host cells and parasite at any one time will be proportional to the amount entering from the alimentary canal.

The fuller interpretation which was desired of the results being obtained in the oral test against *P. gallinaceum* was therefore considered to be dependent on the assessment of the contribution made by (a) the rate of absorption of the drug, (b) the ability of the host to combine with or remove the drug before it enters into contact with the parasite, and (c) the intrinsic activity of the drug. To obtain absolute values for (a) and (b) requires an accurate investigation of the drug, using delicate methods of estimation, and although this was done for as many substances as possible by Dr. A. S. Spinks, a colleague in these laboratories, his facilities were not sufficient for him to cope with all of them, and we were thrown back on biological methods to give relative values. Thus the ratio of the acute oral median lethal dose to the acute intravenous median lethal dose gives a good idea of the relative rate of absorption of a substance. If a convenient standard is chosen—mepacrine was ours—the substance being studied can be related to it in an approximate way. To measure (b) biologically entails removing absorption as a variable and giving the drug intravenously in one or more treatments by rapid injection. It is important that the injection be rapid and not continuous, for otherwise all that is accomplished is the substitution of variable absorption by what is the equivalent of continuous and even absorption. Under such circumstances a drug may be excreted or degraded or removed in some other way very rapidly, and yet, because of the continuous supply, it is possible for the parasite to be continually bathed in it.

The effect of the host's mechanisms for dealing with the drug on the result which is obtained following rapid injection can be assessed if the intrinsic activity of the drug is known. Research on the latter was handicapped by lack of apparatus and is only now beginning, but even so the results which have been obtained by the intravenous treatment appear to be significant and are given below in Table VI. They were obtained in a simple way. Chicks inoculated in the usual manner with 50 million parasitized red cells were treated once daily on each of the next three days with a rapid (five seconds) intravenous injection of the particular quantity of drug. The density of the infection was assessed as in the oral test on the morning of the fifth day.

The interest of the results in Table VI is apparent when the results of the oral test are recalled. In the oral test mepacrine, quinine, 3349, 3502, 3711, 3979, 4316 and 4410 are all approximately equally active, while 4430 is about twice and Paludrine is about eight times as good as mepacrine. In the intravenous test, on the other hand, mepacrine, 4316 and Paludrine are about equally active, and then, in order of descending activity, come (i) 3349, (ii) 4430 and quinine, (iii) 3502 and 3979 and (iv) sulphadiazine. The difference between the

TABLE VI
Activity of substances in the intravenous test against *P. gallinaceum*

Substance	Dose, mgm./50 gm. once daily for 3 days							M.E.D.
	2 mgm.	1.4 mgm.	1.2 mgm.	1.0 mgm.	0.8 mgm.	0.6 mgm.	0.4 mgm.	
Sulphadiazine	322/373			427/410				>2 mgm.
Mepacrine ...					1.3/382	18/385	94/410	0.6 "
Quinine ...		6/293	17/293	43/293	116/383	202/381		1.2 "
3349 ...				6/354	31/376	127/376		0.8-1.0 "
3502 ...		12/294	30/294	111/378	152/378			1.2-1.4 "
3711 ...		31/276	112/276	178/385				>1.4 "
3979 ...			33/402	108/392				>1.2 "
4316 ...						6/355	97/355	0.6 "
4410 ...				103/410				>1.0 "
4430 ...		4/293	10/293	33/314	58/354	221/376		1.2 "
Paludrine ...			2/401	2/401		11/340	73/340	0.6 "

two sets of results can be explained, in part, by the differences in the respective rates of absorption of the substances. A relative value for these rates was obtained, as explained above, by comparing oral and intravenous values of the acute M.L.D. The toxicity tests were done accurately in mice but approximately similar ratios were obtained in chicks. In Table VII the rates of absorption are given actual values relative to mepacrine, which is taken as unity. The values, of course, are only rough approximations, but they serve to indicate the differences between the drugs. In the same table the activity of the drugs measured in the various tests is roughly expressed in terms of the activity of mepacrine. For the sake of completeness results obtained against *P. lophurae* in chicks and *P. cathemerium* in canaries are also included (the tests using these two organisms are described below). Finally, an indication of the results obtained in human malaria with the different substances is given as well. The treatments quoted for human malaria would all bring clinical symptoms under control by at least the fourth or fifth day, but they are not necessarily the best to be followed. They do, however, serve to indicate the relative activity of the drugs, and it can be taken that any variation from them, such as the inclusion of a loading dose, or a more intensive treatment, has some special purpose in view, such as quicker control of the fever or a more lasting effect.

TABLE VII
Comparison of antimalarial drugs in various tests

Substance	Activity in oral tests			Activity in intravenous test against <i>P. gallinaceum</i>	Rate of absorption	Activity in human malaria
	<i>P. gallinaceum</i>	<i>P. lophurae</i>	<i>P. cathemerium</i>			
Mepacrine ...	1	1	1	1	1	100 mgm. t.i.d.
Paludrine ...	8	8	0.4	1	10	25 " b.i.d.
4430 ...	2	1	Inactive	0.5	5	200 " t.i.d.
3349 ...	1	Inactive	0.2	0.7	1	200 " "
Quinine ...	1	1		0.6	2-3	600 " "
3502 ...	1	0.5	0.2	0.4	1	Failed at 200 mgm. t.i.d.
Sulphadiazine	0.5-1	0.5-1	Inactive	<0.25	4(?)	1-2 gm. every 4-6 hours

If, for the present, we discount the specificity of action of drugs, and assume that activity against *P. gallinaceum* means activity in human malaria, then the results in Table VII suggest the following conclusions :

(a) The activity of a substance measured in the intravenous test against *P. gallinaceum* appears to be a better guide to the magnitude of the dose which will be required for the treatment of human malaria.

(b) If a drug is not very good in the intravenous test, the dose in human malaria has to be somewhat high and preferably the drug should be favoured by rapid absorption, e.g., quinine, 4430. If it is not favoured by absorption, even a comparatively high dose has little chance of achieving success, e.g., 3502.

(c) A drug which is very poor in the intravenous test may require comparatively enormous doses in human malaria, e.g., the sulphonamides.

(d) A drug which is not only very good in the intravenous test but is also favoured by absorption will achieve an effect at small doses, e.g., Paludrine.

It should be emphasized that none of these statements is made in any sense dogmatically. All that is being done is to give an account of the work which has been going on in this laboratory and to state the hypotheses which, rightly or wrongly, have been followed. Quite clearly, many of the points have to be checked in detail, but because this might take a long time it is felt that it would be better to place the results on record so that other workers may observe how they accord with theirs.

Some experiments which have been done suggest that the great differences in effect observed when drugs, which are apparently equally active orally, are administered by rapid intravenous injection are due to differences in the rate at which the host eliminates or inactivates them, coupled with the rate at which they penetrate the red corpuscles, rather than to differences of intrinsic activity. Even if this is true it is, of course, unjustifiable to assume that the fate of a drug in the chick is the same as in the human being. However, as a generalization it can probably be accepted that the fate of a drug in one of the species of higher animals is at least an indication of its fate in another species, although the details may vary. Thus, although Paludrine is absorbed faster by some species, e.g., the mouse, than by others, e.g., the rat, it can be said as a generalization that it is absorbed quickly by all of them; similarly the rabbit 'has a remarkable ability to destroy quinine' (Burton and Kelsey, 1943), but it seems that all the higher animals destroy it at least fairly rapidly. Doubtless, biology being what it is, exceptions to the generalizations which have been made will be encountered, but unless substances are tested from start to finish in human beings—and such a procedure is more or less impossible—generalizations of one sort or another have to be made and chances therefore taken.

Why it should be the results of the intravenous test rather than the oral test which appear to give a better indication of the size of dose required in human malaria is, as yet, unexplained; an investigation of the problem is being made.

THE SPECIFICITY OF ACTION OF DRUGS

Throughout the discussion of the results of the intravenous test it was assumed for simplicity that activity against *P. gallinaceum* and the species of *Plasmodium* parasitizing man run parallel, but a study of Table VII alone makes it clear that there is a disconcerting variability in the susceptibility of different species to the same drug. It is possible, therefore, for magnificent results to be achieved by a substance against *P. gallinaceum* in every

test, and yet it might fail in one or more of the types of human malaria. Such a result would be a disappointment, but a more disquieting possibility is that one might miss a drug which would be active in human malaria because it is inactive against *P. gallinaceum*.

The reality of the problem thus set to a laboratory attempting to discover new drugs for human malaria by the use of avian malaria was forcibly impressed upon us by the results obtained with 3349, to which earlier results obtained with the sulphonamides added their weight. 3349, which is effective clinically in all types of human malaria, was discovered by its action on *P. gallinaceum*. It is, however, inactive at maximum tolerated doses against *P. lophurae*, and it would therefore have been missed had this species been used as the testing organism. Later, 4430 was discovered, and this substance, again effective in all types of human malaria, is active against *P. gallinaceum* and *P. lophurae*, but inactive against *P. cathemerium*.

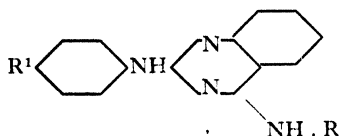
It can be argued that many of the substances which show marked inequalities of action against different species are not of great value in human malaria, and that little would have been lost if they had not been found. Such, for example, are the sulphonamides, neoarsphenamine and 4:4'-diamidino diphenyl stilbene. 3349 and 4430—particularly 4430—are perhaps the nearest approaches to really good antimalarial drugs which might have been missed through the problem of specificity of action; but, even accepting the fact that the ideal antimalarial drug, or an approximation to it, will have all-round activity and would be found by using any species as a test organism, yet some of our recent work has again impressed upon us the necessity for taking specificity of action into account. Thus the formulae of 4430 and Paludrine (the constitutions have been given in Table V(b)) differ only by a CH_3 group, but, whereas Paludrine has an action on every species against which it has been tried, 4430 is inactive at maximum tolerated doses against *P. cathemerium*. Now it happened that many of these biguanide substances had been tested before Paludrine was made, and it is conceivable that, if *P. cathemerium* had been used as the sole test organism, work in the series would have been discontinued and Paludrine would not have been discovered. It has also been shown that 4430 and Paludrine possess activity against exo-erythrocytic forms (Curd, Davey and Rose, 1945b), but again, in the case of 4430, only against those of *P. gallinaceum*. At the time of writing there is every reason to believe that Paludrine is a superior drug to mepacrine both for the treatment and for the prevention of human malaria, and, clearly, to have been so close to its structure with a substance such as 4430 and then to have stopped further work because of apparent inactivity, would have been little short of a calamity.

It is probable that the only really satisfactory way of overcoming the problem of specificity of action is to test every substance against at least two species, which is the procedure adopted by the Board for the Co-ordination of Malarial Studies in the U.S.A. (private communication from Dr. E. K. Marshall), but again we were compelled to compromise, and we have tested only a selection of substances against a second species. The selection has been made along the following lines:

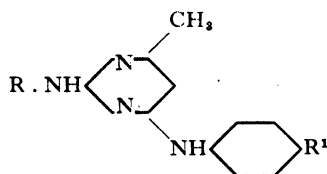
(a) As many completely inactive substances as possible in the test against *P. gallinaceum* have been tested against a second species, either *P. lophurae* or *P. cathemerium*.

(b) Substances which show some activity against *P. gallinaceum* are always tested against *P. lophurae* as well. Such substances will usually be members or forerunners of a series, and one of two courses will then be followed. (i) If the substance has minimal activity against *P. gallinaceum* but is found inactive against *P. lophurae*, then, if chemical

development of the type is proceeding, further testing of the series against *P. lophurae* is discontinued until the activity against *P. gallinaceum* has been increased. (ii) If the substance is found to have some activity against both *P. lophurae* and *P. gallinaceum*, further testing of the series is done entirely against *P. gallinaceum*, and it is assumed that the alterations in structure which improve or impair the activity against *P. gallinaceum* would have the same effect on the activity against *P. lophurae*. This second assumption has been checked fairly completely in two series of substances, the 3979 series with the general formula



and the 4316 series with the general formula



and has held good in both of them (these results will be published elsewhere). The nature of the assumption demands that each species of parasite should be tested against in the same species of host, otherwise differences in the physiology of two species of host might confuse the picture. For this reason *P. lophurae*, like *P. gallinaceum*, has been tested against in six-day-old chicks.

It will have been observed that mention is made of the chemical development of a 'type.' It must be admitted, however, that it is extremely difficult to decide which of the modifications of a substance which are prepared are best considered as variations of a type and which as new types, and it is this which makes the application of the procedure described above a debatable one. For our purposes it is considered that a change, say, in the length of the basic side-chain of a substance, or a change in a single constituent in the nucleus, are variations within the type; on the other hand, a change in the position of a substituent constitutes a change of type.

It should be emphasized, perhaps, that we consider that outstanding activity against any species entitles a substance to be considered for trial in human malaria. It so happens that all the highly active substances discovered in this laboratory have not been more active against a second species than they have been against *P. gallinaceum*, but this result has been entirely fortuitous and should not be taken to imply that marked activity against *P. gallinaceum* is regarded as the *sine qua non* of an antimalarial drug.

THE TEST AGAINST *P. lophurae* IN CHICKS

P. lophurae, which was discovered by Coggeshall (1938) in a Borneo pheasant in the New York Zoological Gardens, can be transmitted to ducks, chicks, turkeys, pheasants and canaries. In this laboratory it has been used for special purposes in ducks and in canaries, but for the most part it has been tested against in chicks, because it was desired, for reasons already given, that the test against *P. lophurae* should be as similar as possible to the test against *P. gallinaceum*.

The strain which was used throughout the experiments was kindly supplied by the Board for the Co-ordination of Malarial Studies, U.S.A. The test is as follows :

(a) The chicks are selected and arranged in exactly the same way as in the test against *P. gallinaceum*.

(b) They are inoculated intravenously with 60–100 million parasitized red cells. *P. lophurae* in chicks is not as virulent as *P. gallinaceum*, and consequently the peak of the parasitaemia is reached more slowly and is not so high. Even with big inocula the peak is not reached until the sixth or seventh day of the test ; without them it is difficult to obtain a fair uniformity amongst the control birds.

(c) The first dose of drug is given orally by means of a catheter-tube about four hours after inoculation, and the treatment is repeated twice daily on each of the next four days.

(d) Assessment of activity is made in exactly the same way as in the test against *P. gallinaceum*.

The differences in detail between the tests against *P. gallinaceum* and *P. lophurae* are a consequence of the comparative benignity of the infection with *P. lophurae* in chicks. The delay before the peak of the parasitaemia is reached means that one more day's treatment is given, and at the peak the density of the infection is not as great as with *P. gallinaceum*. Consequently, a drug is somewhat favoured in this test in comparison with the task set it against *P. gallinaceum* ; in other words, if a substance were equally active against *P. gallinaceum* and *P. lophurae* the results of the two tests, achieved by the methods described here, would make it appear slightly more active against *P. lophurae*. If this point is remembered a satisfactory interpretation of the test can be made. As an example of the results which are obtained the details of some obtained with mepacrine are given in Table VIII. It can be seen that an assessment of activity can be made, and an M.E.D. derived, along the lines described for *P. gallinaceum*. The activity against *P. lophurae* of some of the substances mentioned in this paper has already been quoted in Table VII ; the activity of the remainder can be summed up as follows : mepacrine, pamaquin, 3979 and 4316 are about equally active against *P. lophurae* and *P. gallinaceum*, 3711 is about one-third to one-half as active against *P. lophurae*, and 4821 is inactive against this species.

THE TEST AGAINST *P. lophurae* IN CANARIES

This infection was not standardized to serve as a possible test infection, but a few observations were made which may be of interest. There were two main reasons for transmitting *P. lophurae* to canaries. In the first place, we wondered whether the canary would serve as a more satisfactory host than the chick or the duck in which to conduct causal prophylactic experiments (these experiments will be described in the second paper in this series). Secondly, it was desired to determine the effect of 4430 on *P. lophurae* in canaries. It has already been remarked that this substance is active against *P. gallinaceum* and *P. lophurae* in chicks, but apparently inactive against *P. cathemerium* in canaries, while a very closely related substance, Paludrine, is active in all three infections. It was considered that the failure of 4430 against *P. cathemerium* could be due to some change produced in the substance by the canary, rather than to lack of the requisite activity, and to check this possibility an infection with a malarial parasite known to be sensitive to 4430 was required in this host. Accordingly, *P. lophurae* was introduced into it.

Nine canaries were each injected with approximately 40 million parasitized red cells drawn from a chick infected with *P. lophurae*. Only a small number of parasites (about 1 per 50 oil-immersion fields of a thin smear) could be seen on the fifth day of the experiment, but afterwards the density gradually increased until on the ninth day about 1 parasite per field could be found. Three birds were then treated with 4430, 1 mgm. per 20 gm. b.i.d., three with 4430, 0.5 mgm. per 20 gm. b.i.d., and three were kept as controls. The control infections rose to a peak (10–20 parasites per field) on the 11th and 12th days and then gradually declined; the infection in the treated birds, on the other hand, was quickly brought under control and reduced within two days to a low level. It was

TABLE VIII

Activity of mepacrine against *P. lophurae* in chicks. (Inoculum 70 million parasitized red cells; treatment oral b.i.d. $\times 4\frac{1}{2}$; doses in mgm./50 gm.; density of infection as number of parasitized corpuscles in 500 examined)

Dose, mgm./50 gm. b.i.d. $\times 4\frac{1}{2}$	Density of infection at specified day			
	5	6	7	8
4 mgm.		1	0	0
		0	0	0
		1	0	0
		2	0	0
		1	0	0
		3	0	0
2 mgm.		4	1	0
		2	2	0
		8	2	0
		4	0	0
		10	3	1
		17	1	0
1 mgm.		182	249	232
		54	124	186
		31	11	6
		6	1	4
		16	4	11
		11	12	63
Controls	87	195	359	236
	84	227	221	126
	43	116	285	320
	38	148	256	308
	25	99	232	364
	8	36	144	85

M.E.D. = 2 mgm./50 gm.

shown, therefore, that 4430 is effective against *P. lophurae* in canaries; but having determined this fact the work was discontinued, because it seemed that the infection was too benign and the experiments with it too prolonged for it to be of general use.

THE TEST AGAINST *P. cathemerium* IN CANARIES

Information was sometimes required concerning the activity of substances against *P. cathemerium* or *P. relictum* in canaries. It was only rarely that the latter species was used, and all the experiments recorded below were done using the M strain of *P. cathemerium*, which we obtained through the kindness of Dr. R. G. Coatney, of the United States Public Health Service.

The most common way of using a malarial infection of canaries for testing antimalarial drugs is to follow the procedure laid down by Roehl (1926). He inoculated his experimental birds into the pectoral muscle with parasitized red cells, and treated them with the test substances orally daily for six days. In untreated canaries, infected in this manner, parasites can usually be found on the fourth to eighth days within three minutes' examination of a stained blood smear under the oil-immersion lens, although, of course, a more extended examination would reveal them earlier. The comparatively lengthy time taken for the infection to become patent is due to the few parasites which succeed in obtaining an entry into the blood-stream; the difficulties impeding entry are reflected in the irregularities exhibited in the course of the infection in different birds.

The prepatent period is still further lengthened if an infected bird is treated with an antimalarial drug, and it was upon this basis that Roehl designed his test. We, however, wished that the test in the canary should simulate the tests in the chick as closely as possible, and clearly the first requisite for this was the intravenous inoculation of parasites, so making the infection more acute. The steps in the test, as it was finally worked out, are as follows.

(a) The canaries used in the experiments weigh 18-22 gm.; three or four are included in each group.

(b) Blood drawn from a canary at the height of its infection is diluted with citrated saline until it contains 8-12 million parasitized cells in each 0.2 ml.

(c) The inoculum is given intravenously into one of the toe-veins; 0.2 ml. is given to each bird. Injection into a toe-vein is not as difficult as might be imagined, and is followed by much less bleeding than after injection into the leg-vein. A no. 20 Record fitting needle is used, and little more than the point is inserted into the vein.

(d) The drugs are administered orally in solution or suspension through a catheter-tube passed into the gizzard; 0.5 ml. is allowed for each 20 gm. bird. The first dose is given about four hours after inoculation and is repeated twice daily on each of the next four (occasionally three) days.

(e) The density of the infection is estimated in terms of the number of parasites per microscopic field, using a 1/12 inch oil-immersion lens and $\times 6$ ocular. The peak of the parasitaemia usually occurs on the fifth or sixth days of the test.

Some of the results which have been obtained are given in Table IX, which is divided into two parts. The first part contains the complete data of an experiment with mepacrine and exemplifies the ease with which the test is read. The second part lists most of the substances previously mentioned in this paper, and gives sufficient information to allow a comparison of their activity against *P. cathemerium* to be made; the readings for the parasitic density which are quoted in this part are the extremes encountered in each particular group.

A study of Table IX reveals several points of interest:

(a) It will be agreed that the course of the infection in untreated birds is satisfactorily uniform, and that the contrast between the results obtained in birds treated with an active drug and in the controls is very marked.

(b) Slight activity on the part of a substance is indicated, as in the tests in the chick, by a lack of uniformity in the results obtained in the different birds of a single group. The lowest dose giving a uniformly low result is taken as the M.E.D.

(c) While the activity of 3349, 3502, 3711, 3979 and 4316 against *P. cathemerium*

TABLE IX (a)

Activity of mepacrine against *P. cathemerium* in canaries
(Inoculum 8 million parasitized cells; treatment b.i.d. $\times 4\frac{1}{2}$)

Dose, mgm./20 gm. b.i.d. $\times 4\frac{1}{2}$	Density of infection			
	3	4	5	6
Controls	1/1 1/1 1/1	5/1 10/1 5/1	10/1 20/1 15/1	20/1 15/1 15/1
0.8 mgm.	1/10 1/50 1/10	1/20 1/50 1/25	1/30 1/50 1/10	1/5 1/5 1/5
0.4 mgm.	1/10 1/5 1/10	1/5 1/5 1/10	1/3 1/5 1/3	1/1 1/3 2/1
0.2 mgm.	1/3 1/5 1/2	1/1 1/2 1/2	1/5 1/2 2/1	1/5 1/2 3/1
0.1 mgm.	1/10 1/2 1/1	2/1 2/1 5/1	5/1 2/1 10/1	10/1 10/1 15/1
0.05 mgm.	1/3 1/1 2/1	5/1 10/1 5/1	25/1 15/1 10/1	20/1 20/1 15/1

M.E.D. = 0.2 mgm./20 gm.

TABLE IX (b)

Activity of various substances against *P. cathemerium* in canaries

Substance	Dose, mgm./20 gm.	Density of infection			M.E.D.
		4	5	6	
Controls ...		5/1 - 12/1	10/1 - 20/1	20/1 - 25/1	
3349	2.0 mgm.	1/50- 1/50	1/50- 1/50	1/50- 1/50	1.0 mgm./20 gm.
	1.0 "	1/50- 1/5	1/50- 1/5	1/50- 1/5	
	0.5 "	1/5 - 1/2	1/1 - 10/1	5/1 - 20/1	
3502	2.0 mgm.	0/50- 1/50	1/50- 1/10	1/50- 1/5	1.0 mgm./20 gm.
	1.0 "	1/50- 1/15	1/50- 1/5	1/50- 1/5	
	0.5 "	1/5 - 1/2	1/10- 8/1	1/10-10/1	
3711	1.0 mgm.	1/5 - 3/1	1/1 - 5/1	1/5 - 5/1	
3979	1.0 mgm.	1/50- 1/5	1/50- 1/10	1/50- 1/5	
4316	1.0 mgm.	1/50- 1/50	0/50- 1/50	0/50- 1/50	1.0 mgm./20 gm.
	0.5 "	1/50- 1/5	1/50- 5/1	1/25-10/1	
4430	1.0 mgm.	5/1 - 10/1	15/1 - 20/1	20/1 - 25/1	Inactive
Paludrine ...	1.0 mgm.	0/50- 1/50	0/50- 1/50	0/50- 1/50	0.5 mgm./20 gm.
	0.5 "	0/50- 1/10	1/50- 1/10	0/50- 1/10	
	0.25 "	1/1 - 5/1	1/1 - 10/1	5/1 - 20/1	
Pamaquin (bis-naphthoate)	0.02 mgm.	0/50- 1/50	0/50- 0/50	0/50- 1/50	0.02 mgm./20 gm.
	0.01 "	0/50- 1/1	1/50- 5/1	1/50-10/1	

is approximately the same as against *P. gallinaceum*, that of mepacrine is much greater and of Paludrine much less against *P. cathemerium*. Thus the M.E.D. of the first five drugs measured against *P. gallinaceum* is 2 mgm. per 50 gm. and against *P. cathemerium* is about 1 mgm. per 20 gm., results which are roughly of the same magnitude. The M.E.D. of mepacrine, however, against *P. cathemerium* (0.2 mgm. per 20 gm.) appears to make it about five times more active against this species than against *P. gallinaceum* (M.E.D. 2 mgm. per 50 gm.), while Paludrine is about five times less active against *P. cathemerium* (M.E.D. about 0.5 mgm. per 20 gm. compared with 0.25 mgm. per 50 gm. against *P. gallinaceum*). Whether these great differences are due to the hosts or to the parasites remains to be determined, but at least it can be said that since both 3349 and Paludrine are efficacious in human malaria, the one at twice the mepacrine dose and the other at less than half this dose, then the activity of mepacrine against *P. cathemerium* might be an unduly exacting standard for the assessment of the activities of antimalarial drugs.

It appeared of interest to compare results obtained in this method of testing substances against *P. cathemerium* with those which would be obtained if Roehl's procedure were followed. It has been mentioned that Roehl interpreted activity in terms of a lengthened prepatent period. In untreated birds parasites can be found easily on the fourth to eighth days, while in birds treated with an active drug during the first few days (usually six) their appearance is delayed. Roehl fixed on delay to the tenth day as being significant. It sometimes happens, however, that a Roehl test gives results which are not easy to interpret. In a single group the prepatent period in one or more birds may fall within the control times, while in others it will be intermediate, perhaps nine days, or possibly just barely ten days. Whether such a result should be ascribed to very slight activity on the part of a drug is often difficult to decide, particularly because of the small number of canaries which economic considerations dictate should be used in the experiments. Activity at this level appeared, therefore, to be very suitable for comparing the sensitivity of the present test and the Roehl tests, and it was deliberately sought by varying the size of dose of a substance known to be active. The results are quoted in Table X. It will be apparent that, because of the uniformity of the untreated infection, the interpretation of our test is much more unequivocal than that of the Roehl test.

THE SEQUENCE FOLLOWED IN THE SELECTION OF SUBSTANCES FOR TRIAL IN HUMAN MALARIA

It will be advantageous, perhaps, now that a detailed consideration has been given to the methods used in this laboratory for the measurement of antimalarial activity, if the steps in the procedure which is followed in the final selection of substances for trial in human malaria are enumerated.

1. All substances are tested orally against *P. gallinaceum*; active substances are assayed to a critical dose region which allows an approximate M.E.D. to be calculated for them.

2. As many inactive substances as possible are retested against *P. lophurae* in chicks or *P. cathemerium* in canaries. In this way it is hoped that the problem of specificity of action will be overcome.

3. When a substance has activity against both *P. gallinaceum* and *P. lophurae*, and an attempt is being made to augment it, the assumption is made that the activity of the variations of the substance, so long as they keep to type, run parallel against the two species, provided that they are tested against in the same species of host. The difficulty here lies in deciding what constitutes a variation of a type and what is a new type.

4. The exhibition of high activity against any species justifies considering a substance for trial in human malaria, although it has happened that all the compounds tested in this laboratory so far have been at least as active against *P. gallinaceum* as against *P. lophurae*. High activity is defined as activity on a par with that of a successful drug such as mepacrine or quinine.

5. Substances with high activity in the oral test against *P. gallinaceum* are also tried in the intravenous test against this species. What seem to be important differences between various antimalarial drugs have been demonstrated by the use of the intravenous test,

TABLE X

Comparison of the Roehl test with the present test against *P. cathemerium*.
Both series of birds given same inoculum and treated with the same solutions. Series I, present test ;
Series II, Roehl test

Treatment	Series I Inoculation of parasites intravenously				Series II Inoculation of parasites intramuscularly								Interpre- tation of result
	Density of infection			Interpre- tation of result	Density of infection								
	4	5	6		5	6	7	8	9	10	11	12	
Controls ...	10/1 2/1 10/1 5/1	20/1 15/1 20/1 15/1	10/1 5/1 10/1 15/1		1/50 1/20 1/10 —	1/1 1/15 5/1 —	10/1 5/1 10/1 1/50	20/1 15/1 15/1 1/15	10/1 10/1 5/1 5/1	10/1 10/1 5/1 10/1	5/1		
3502 0.5 mgm./20 gm. b.i.d. $\times 5\frac{1}{2}$	1/2 5/1 3/1 1/2	2/1 20/1 2/1 1/2	1/5 10/1 5/1 1/1	Slightly active	— 1/1 — —	1/50 2/1 — —	1/1 1/1 — —	5/1 1/10 — —	10/1 1/10 1/50 1/50	15/1 1/10 1/50 1/20		1/20 1/1	Doubtful
3502 1.0 mgm./20 gm. once daily for 6 days	1/5 1/10 1/5	1/5 1/10 1/2	1/2 1/10 2/1	Active	— — —	— 1/50 —	— 1/50 1/50	— 1/50 1/10	— — 3/1	— — 7/1	— — 10/1	1/50 — 15/1	Doubtful

and substances which show promise in it are given priority in the trials in human malaria.

6. Some toxicity tests are necessarily done in birds, but accurate ones are done only in mammals, usually rats and mice. These toxicity tests, of course, are an essential preliminary to trials in human beings, but they also serve to give a very good idea of the rate of absorption of a substance.

7. The final appraisalment of the chances of a substance having activity in human malaria takes into account oral activity, intravenous activity, specificity of action, rate of absorption, and toxicity.

SUMMARY

1. A description is given of tests for antimalarial drugs using *P. gallinaceum* in chicks, *P. lophurae* in chicks, and *P. cathemerium* in canaries. In all these tests a standardized number of parasitized corpuscles is injected intravenously into the experimental birds

and treatment is given orally twice daily. The assessment of activity is made by comparing the density of the infection in treated and untreated birds at the time when the peak of the parasitaemia curve is reached.

2. The factors which may influence the net results recorded in the tests are given, together with a description of the attempts which were made to evaluate each one separately. Particular attention is paid to the significance of the results achieved following the rapid intravenous injection of test substances.

3. The problem of specificity of action of antimalarial drugs is discussed, and examples are given to show why the problem must be taken into account.

4. The use of the tests for the discovery of substances effective in the control of clinical symptoms in human malaria is considered, and the steps which are taken in the evaluation of a substance are described.

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THE SEX RATIO OF INFECTED FLIES FOUND IN TRANSMISSION-EXPERIMENTS WITH *GLOSSINA MORSITANS* AND *TRYPANOSOMA RHODESIENSE*

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The single strain of *Trypanosoma rhodesiense* being studied at Tinde laboratory, Tanganyika Territory, is the one which was isolated by Corson (1936). Since 1940 it has been passaged cyclically through various animals—chiefly sheep, reedbuck, Thomson's gazelle and *Cercopithecus* monkey, with occasional passages through other species. The experimental procedure followed is described elsewhere in these *Annals* (Burtt, 1946). Two categories of *Glossina morsitans* were used: flies which emerged from pupae left under normal laboratory conditions, and those which emerged from pupae incubated at approximately 30° C. The infection-rate with *T. rhodesiense* in the latter was considerably higher than in the former, and a significantly higher percentage survived to be examined of those from incubated pupae than of those from untreated pupae. Numbers of infected flies in both categories were evidently not detected by modifications of Lloyd and Johnson's dissection technique, and therefore all rates of infection were based solely on flies examined by the isolation method.

At an early stage in the course of this work it was remarked that very many more male infected flies were being obtained than females. Records of the sexes of all flies used in experiments were therefore kept, so that this matter could be investigated. Table I shows the percentage of infected male and female flies found in isolation-experiments from normal untreated pupae and from pupae incubated at approximately 30° C. The results are from the four species, sheep, reedbuck, Thomson's gazelle and monkey. The last line of the table shows the combined results from all species studied.

The numbers of male and female flies used were approximately equal. The results were examined statistically, and it was found that there was a significantly greater rate of infection in male than in female flies from each host species. The sex ratio of infected flies did not differ significantly in the two categories of pupal treatment, either between the species of host used or in the total results. Significantly fewer females than males survived to be examined in every case, and the ratio of survival of the sexes did not differ significantly between the different host species.

DISCUSSION

There are few published records of the proportion of male and female flies found infected in transmission-experiments. Corson (1935) recorded one experiment from reedbuck (*Redunca arundinum* Boddaert); he found 20 male and 5 female salivary-gland-infected flies in 40 males and 33 females dissected. There is an evident preponderance of infected males in these results, but the numbers are too small to be significant.

Duke (1930) gave data for *T. rhodesiense* and *T. gambiense* in relation to *G. palpalis*. The results were as follows:

	Male	Female
No. of flies examined	12,737	11,772
No. with flagellates (including gut infections)	581 (4.56 per cent.)	656 (5.57 per cent.)
No. with infected salivary glands	178 (1.398 per cent.)	183 (1.554 per cent.)

Re-examination of the data led Duke (1933a) to qualify his original statement that there was no difference in the infection-rate in the two sexes. When the total number which contained flagellates (including gut infections) was examined statistically there was found to be a significantly higher proportion of females infected than males ($P = 0.01$). He expressed the view that this might be due to the fact that the females fed more readily and tended to survive better than the males. However, Dr. Jackson, Entomologist of the Tsetse Research Department, at the present writer's request, examined the results in relation to the numbers of salivary-gland-infected flies recorded, and no significant difference was found between the two sexes.

When the disparity between the percentages of male and female infected flies in the Tinde experiments was first remarked, it was thought that it provided a straightforward demonstration that female *G. morsitans* were less infectible with *T. rhodesiense* than males; but the fact that more female flies were dying than males suggested that the latter factor might have played the decisive rôle in determining the results obtained. It was evidently essential to consider both the proportion in which the sexes emerged and the sex ratio of survival of the flies in the experiments.

With regard to the proportion in which male and female flies are produced, Potts (1933) recorded exact equality in 262 *G. morsitans* which emerged from bred pupae at Kikori. Frequently, however, there tends to be a slight preponderance of females over males. Nash (1930) stated that, out of 1,040 *G. morsitans* which emerged from wild pupae at Kikori, 53 per cent. were females. On the other hand, the same author (1933) recorded 5,168 wild puparia of *G. morsitans* as having been collected at Kikori up to that time, and 50 per cent. of the flies which emerged were females. The Department of Tsetse Research, Shinyanga, has considerable unpublished data on this subject, and Mr. W. H. Potts, Entomologist, states that: 'I think it is fair to say they (the sexes) do emerge in very closely equal numbers'.* As examples of emergences from wild pupae he gives the following figures:

<i>G. morsitans</i>	15,392 males	15,990 females
<i>G. palpalis</i>	4,022 "	4,134 "

Between July, 1942, and June, 1944, the numbers of male and female *G. morsitans* which emerged at Tinde laboratory were as follows:

	Male	Female
From pupae left under normal laboratory conditions ...	9,588	9,720
From pupae incubated at approximately 30° C. ...	6,049	6,158

* The writer is indebted to Mr. Potts for permission to quote this personal communication.

The slight preponderance in the numbers of female flies is not significant.

The results at Tinde thus conform with those of other workers: male and female flies were produced in approximately equal numbers. It is of interest that incubation of pupae at approximately 30° C. did not affect the proportion in which the sexes emerged.

With regard to the survival of the two sexes, the records show that the females are generally longer-lived than the males, both in nature and under suitable laboratory conditions. Thus Jackson (1941, 1944), in an analysis of a population of wild *G. morsitans* at Kakoma, found that, in relation to the numbers marked and the numbers caught on recapture dates, the recaptures of females were fewer than those of males and did not show nearly such a rapid decline at successive dates after marking. This suggests that the female population of flies is larger than the male, while the death-rate in the females must be lower. Also, if the mean length of life found from the marked flies was anywhere near the true value for unmarked flies, then the female flies must live at least twice as long as the males, otherwise (assuming a 50 per cent. mortality in the pupae) the species would become extinct. It was not, however, suggested that the females are inherently longer lived, the greater death-rate in males being ascribed to hazards of various kinds following their more active life. Other investigators have shown that female flies live slightly longer than males under certain experimental conditions. Nash (1936) found that this applied to both *G. tachinoides* and *G. submorsitans*. He kept the flies protected from sun and rain in a wall-less grass-roofed hut situated under a tree. Jack (1939) records similar results in respect to *G. morsitans*. In this case the flies were kept (four males together with six females) in perforated zinc cylinders under a variety of temperature and humidity conditions.

Turning now to the mortality ratio which occurred in the Tinde experiments with *G. morsitans*, it was found that there was a consistently higher death-rate in females than in males throughout their maintenance in Bruce boxes. The ratio between the percentage of female and male deaths increased with the experimental age. In flies which had been placed singly in bottles ready for isolation, a proportion died and could not be tested. In these, also, the mortality ratio was similar to that which occurred in the boxes, thus further accentuating the preponderance of males left in those examined. This seemed evidently to be a continuation of the ill effects which had been exerted by maintenance in the boxes. Such a higher death-rate in female *G. morsitans* does not conform with what happens in nature or with other methods of maintenance, and may have caused some, or all, of the disparity in the infection-rates found between the sexes in the experiments. This interpretation may receive some support from the coincidence of the highest ratio between male and female survival with the highest male/female ratio of infected flies. The sex ratio of survival and the sex ratio of infection obtained from sheep, reedbuck, Thomson's gazelle and monkey are shown in Table II. The data have been arranged in accordance with the magnitude of the male to female survival ratio.

In the results recorded by Duke (1930) large numbers of *G. palpalis* were considered, and the salivary gland infection-rate of males and females was found to be the same. On the assumption that the sexes were present in approximately equal numbers at the start, there was a slightly greater death-rate in females than in males. Nevertheless the percentage of female deaths in Duke's experiments was significantly less ($P < 0.01$) than that of female *G. morsitans* in the experiments considered in this paper. Duke (1933*b*, 1933*c*) was using about 50 *G. palpalis* per box. Corson (1937), like the present writer (1946), used about

30-35 *G. morsitans* per box. The results suggest that maintenance in boxes affects the two species differently. Apparently it has little harmful influence on the survival of female *G. palpalis*, but does have a markedly deleterious effect in the case of females of *G. morsitans*.

It was found at Tinde that, in *G. morsitans* which had been isolated singly into bottles and fed, females did live slightly longer than males. This was the only stage in the experiments when the survival ratio was normal. It appears, therefore, that, for a definite answer to be obtained to the question as to whether the two sexes of *G. morsitans* truly differ in their infectibility, work with flies kept and fed individually will have to be undertaken.

TABLE II

The sex ratio of survival and the sex ratio of infection of *G. morsitans* in transmission-experiments

Category of flies	Year	Ratio male/female percentage	
		Survival	Infection
From normal untreated pupae	From July 1942	1.3	1.1
	1943	1.3	3.6
From pupae incubated at approximately 30° C.	1943	1.4	2.2
	1944	1.6	2.6
From normal untreated pupae	1944	1.6	2.6
	To May 1945	2.1	12.9

SUMMARY

In transmission-experiments with *Glossina morsitans* and *Trypanosoma rhodesiense*, the proportion of infected males was greater than that of females by more than 2/1, both in the case of flies which emerged from normal untreated pupae and in those from pupae incubated at approximately 30° C.

The sex ratio of infection did not differ significantly from different vertebrate host species or between either category of flies.

In every case the number of females which survived to be examined was significantly less than that of males. The sex ratio of survival did not differ significantly from different species of infected vertebrate host.

Male and female *G. morsitans* emerged at Tinde laboratory in nearly equal numbers both from pupae left under normal laboratory conditions and from those incubated at approximately 30° C.

There was a higher death-rate in female than in male flies throughout the period of their maintenance in Bruce boxes. The disparity between the female and male death-rate became greater as the experimental age of the flies increased. A proportion of the flies placed singly in bottles ready for isolation died and could not be tested. In these the mortality ratio between the sexes was similar to that found in the boxes and seemed to be a continuation of the ill effects of the box maintenance.

The highest male/female survival ratio coincided with the highest male/female infection ratio.

Since the greater death-rate in females than in males is contrary to what takes place in nature or under other conditions of maintenance, it seems probable that the high mortality-rate in the female flies was primarily responsible for the disparity found in the infection-rate in the two sexes of *G. morsitans* in the experiments.

In Duke's records, the death-rate in female *G. palpalis* seemed to be only very slightly greater than in males, and there was no significant difference in the salivary gland infection-rate between the two sexes. The death-rate in female *G. palpalis* in Duke's experiments was significantly less than that in female *G. morsitans* at Tinde. This suggests that the two species respond differently to experimental maintenance in fly-boxes, *G. palpalis* being better able to withstand mass confinement than *G. morsitans*.

The only stage in the Tinde experiments wherein a normal survival ratio between the sexes occurred was in flies which had been isolated singly into bottles and had fed. For an answer to the question as to whether male and female *G. morsitans* really differ in infectibility with trypanosomes, work with flies kept singly should be undertaken.

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THE EFFECTS OF MEPACRINE ON THE GASTRO- INTESTINAL TRACT OF MAN

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The most frequent toxic effect of mepacrine administration is a disturbance of the gastro-intestinal tract, which varies in severity from a slight diarrhoea to intense nausea, vomiting and severe diarrhoea accompanied by fever and prostration. Fortunately these unpleasant side-effects are seldom seen when mepacrine is given in doses of 0.1 gm. a day. Shortly before the Army Malaria Research Unit began its work, however, diarrhoea and vomiting suddenly occurred on a large scale in North Africa among troops who had recently begun to take suppressive doses of mepacrine, and it was decided to make a study of the effects of the drug on the gastro-intestinal tract. Preliminary experiments were done in animals to determine the relative toxicities of different salts of mepacrine, to estimate the effects of different modes of administration, and to work out the mechanism of the disturbance. These experiments have in part been reported (Army Malaria Research Unit, 1945). Human experiments were then undertaken. Observations were made on a large number of volunteers taking various amounts of mepacrine, and a limited number of cases were submitted to intensive investigation by means of fractional test meals, barium meals, cholecystography, gastroscopy and bacterial examination of the stools. The results of the human experiments are presented in this paper. They show that when mepacrine is given in what is now the common suppressive dose, 0.1 gm. a day, gastro-intestinal disturbances are slight and infrequent, and they suggest an explanation of the serious effects seen after the intermittent dosage used in North Africa in 1943.

TECHNIQUES

Barium Meals. All the women volunteers had two control barium meals before the exhibition of mepacrine. The soldier volunteers had one control meal. There was no preliminary preparation, and the volunteers were on their normal diet the day before the experiment. On the morning of the barium meal they had a cup of tea on awakening. After the three-hour film they were given two cups of tea and some sandwiches, and they resumed their normal diet after the six-hour films. The opaque meal consisted of 1/8 pint of Horlick's Shadow Food mixed with an equal amount of water. All the films were taken with a Lysholm Grid but without the use of a Potter-Bucky diaphragm. Films were taken, after preliminary screening, immediately after the barium meal was given,

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and at 20 minutes, 40 minutes, and 1, 2, 3, 4, 5, 6 and 24 hours. Reductions of the 15" by 12" films were made to facilitate the examination of comparable series.

Gastroscopy. All gastroscopic examinations were made with a Hermon Taylor gastroscope, and there was one control examination before the exhibition of mepacrine. Premedication consisted of hyoscine hydrobromide gr. 1/100 one hour before gastroscopy.

Plasma Mepacrine Estimation. Plasma mepacrine concentration was estimated by a modification of the method of Masen (1943).

Bacteriological Examination of the Faeces. The relative numbers of Gram positive cocci and bacilli and Gram negative bacilli were estimated from stained films. The proportions of coliform bacilli and faecal streptococci were estimated by inoculating McConkey agar plates with tenfold dilutions of a suspension of faeces.*

THE EFFECTS OF LARGE DOSES OF MEPACRINE

Mepacrine in doses from 0.6 to 1.4 gm. was given to 16 women volunteers so that the gastro-intestinal effects of the drug could be studied in a severe form and their characteristics easily identified. Twelve women received a single dose of mepacrine 0.6 to 1.0 gm. one and a half hours before the barium meal. The other four were given 0.6 gm. on the preceding day and 0.2 to 0.8 gm. one and a half hours before the barium meal. Eleven of the subjects had never received mepacrine before; the remaining five had been on a suppressive dosage for several months, but two barium meals which preceded this experiment were normal in each case and were regarded as controls.

Clinical Observations. A regular sequence of symptoms was seen in all but three of the volunteers. During the first two hours after taking the drug there was headache, nausea, and sometimes a single emesis or a loose bowel motion. For the next two to four hours the subjects usually felt well except for a slight headache. After this period of relative well-being, the headache usually became more severe, nausea returned, sometimes accompanied by sudden and repeated vomiting, and colicky abdominal pain and diarrhoea were frequent. In the more severe cases there was fever and prostration. Pallor and coldness of the extremities were the only abnormal physical signs. Eight to nine hours after the administration of the drug, improvement set in and recovery was usually completed in a few hours.

Barium Meals. Radiographically there were abnormalities in the appearance of the stomach or intestines in all but two of the cases. One exception was a woman who received a single dose of 1.0 gm. and felt well until the end of the fourth hour, when she developed a headache. After seven hours she became nauseated and vomited twice. In her case the disturbance occurred after the barium had left the part of the gastro-intestinal tract which was involved, and no radiographic record was obtained. The second exception was one of those who had no symptoms. The other two volunteers who had no symptoms had abnormal radiographic findings of moderate severity.

In the period immediately following the taking of the barium meal, there was gastric hypersecretion, hyperperistalsis, an increase in tone which was most marked in the pyloric antrum, and pylorospasm. These changes often resulted in a reversed-L-shaped stomach, the hypertonic pyloric antrum being at right angles to the body of the stomach. In one case the disturbed tone and motility gave rise to a 'cup and spill' deformity. Pylorospasm

* The bacterial examinations were made by Dr. W. D. Fleming.

was sometimes so severe that no emptying occurred for more than an hour. These changes were followed by a decrease in gastric tone and peristalsis, and this with continued pylorospasm caused delay in final emptying of the stomach.

A disturbance of the pattern of the small intestine was the most frequent abnormal finding in the series. The normal pattern of the valvulae conniventes was replaced in most cases by a fragmentation of the barium into small ragged clumps of irregular size and shape. Sometimes exaggerated segmentation resulted in columns of barium a few inches in length, and the edges of these columns showed irregular smooth indentations. Numerous small flecks of barium were often left in the jejunum and upper ileum after the main mass of the meal had passed on. In the lower ileum there was a combination of rings of contraction and decreased tone in the intestinal wall, which led to the accumulation of barium in round masses a few centimetres in diameter. In one case there were multiple fluid-levels in the small intestine. The rate of passage of the meal through the jejunum and upper ileum was usually much increased, but there was often delay at the ileo-caecal valve before the meal entered the colon. More often there was an ileal residue at six hours which was much greater than that seen in the control meals.

Once the meal entered the colon it usually advanced very rapidly, and in some cases it reached the pelvic colon in six hours. The only other change noted in the colon was an outpouring of secretion. (Scudi, Jelinek and Kuna (1944) observed that in rats dying after the administration of large doses of mepacrine the gastro-intestinal tract was distended with fluid. The Army Malaria Research Unit (1944) with Mr. E. H. Leach showed that in the rat mepacrine stimulates the discharge of the neck mucous glands, the cardiac glands of the stomach and the cells of Brunner's glands in the duodenum.)

Cholecystography. Mepacrine 1.0 gm. caused no radiographically demonstrable disturbance in the function of the gall-bladder in two women volunteers.

Bacterial Flora of the Faeces. There was no significant change in the flora of the faeces of eight volunteers after the exhibition of large doses of mepacrine.

THE EFFECTS OF A SUPPRESSIVE DOSAGE OF MEPACRINE

When the abnormalities which may be caused by mepacrine were thus identified, observations were made on a large number of volunteers who took a suppressive dosage of mepacrine for periods of from a few days to several months. Symptoms were recorded in all volunteers and special investigations were undertaken in a selected number. The results showed that gastro-intestinal disturbances which can be attributed to the administration of a suppressive dosage of mepacrine are infrequent and slight.

Clinical Observations. Out of a group of 85 healthy Oxford women undergraduates, who were on various suppressive régimes and who were closely observed and asked to report all symptoms, no matter how trivial, 12 complained of gastro-intestinal disturbances during periods of mepacrine-administration which covered three to eleven months. The symptoms reported were nausea, vomiting, flatulence, colicky abdominal pain and mild diarrhoea. These occurred in most cases during the first week on the drug and disappeared in a short time despite continued drug-administration. In six cases the organic origin of the symptoms was demonstrated when symptoms immediately disappeared after the substitution of a placebo without the subject's knowledge. Vomiting was seen in only

EFFECT OF LARGE DOSES OF MEPACRINE HYDROCHLORIDE ON THE GASTRO-INTESTINAL TRACT

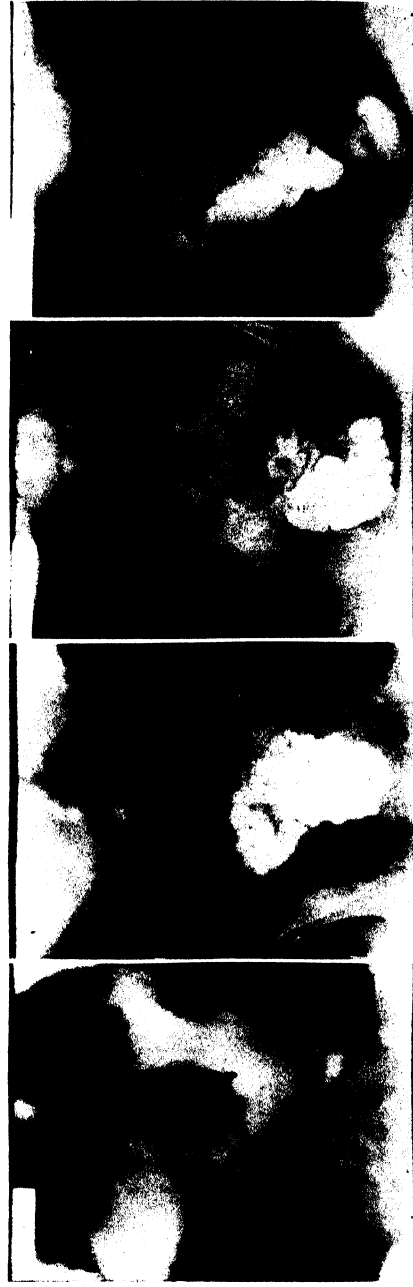


FIG. 1. Control barium meal series on volunteer no. 364.

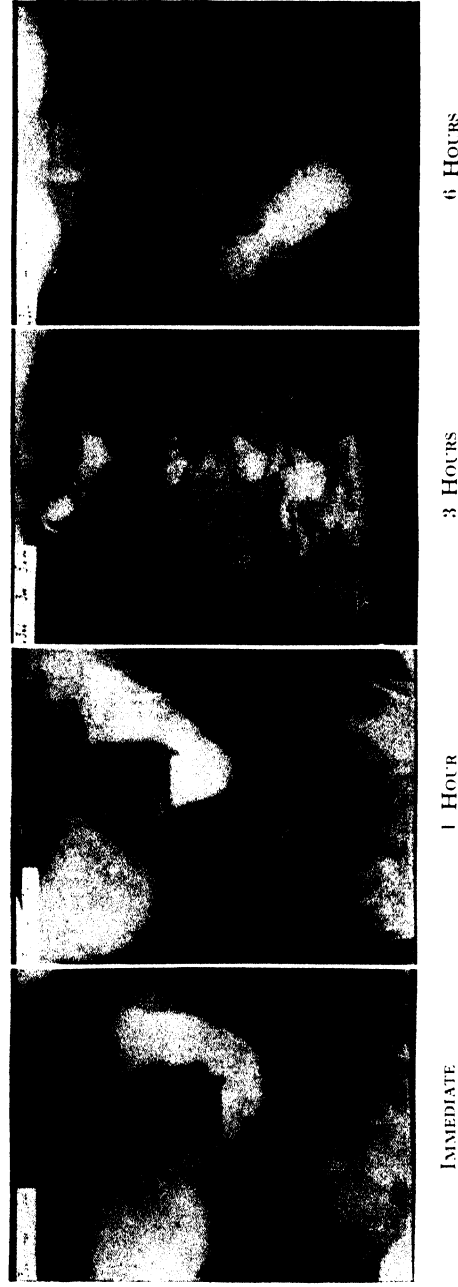


FIG. 2. Barium meal series on volunteer no. 364 after 0.6 gm. mepacrine hydrochloride on previous day and 0.6 gm. mepacrine hydrochloride half an hour before the meal, showing marked delay in start of gastric emptying, increase in gastric secretion, marked irritability of small intestine, and delay in passage into colon.

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EFFECT OF LARGE DOSES OF MEPACRINE HYDROCHLORIDE ON THE GASTRO-INTESTINAL TRACT



FIG. 3. No. 206. Immediately after barium meal. $1\frac{1}{2}$ hours after 1.0 gm. mepacrine hydrochloride taken orally. Marked irritability of stomach producing 'cup and spill' deformity.



FIG. 4. No. 165. Immediately after barium meal. $1\frac{1}{2}$ hours after 1.0 gm. mepacrine hydrochloride taken orally. Increased resting juice, pylorus not open, over-vigorous contraction of stomach indicative of pylorospasm.



FIG. 5. No. 356. 40 minutes after barium meal. 2 hours after 0.6 gm. mepacrine hydrochloride taken orally. Small intestine shows irregular clumping indicative of irritability.

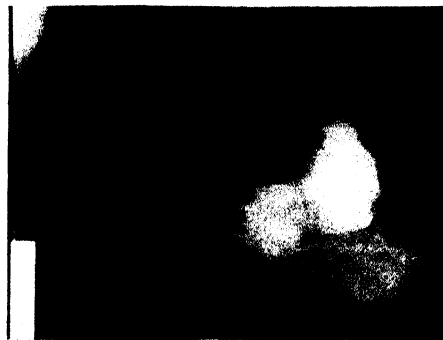


FIG. 6. No. 358. 3 hours after barium meal. $4\frac{1}{2}$ hours after mepacrine hydrochloride taken orally. Barium held up in loop of small intestine, possibly owing to ileal spasm.



FIG. 7. No. 356. Same meal as fig. 4, but 6 hours after barium meal. $7\frac{1}{2}$ hours after 0.6 gm. mepacrine hydrochloride taken orally. Shows extremely rapid advance of meal.

three cases—cases who had been on a régime of 0.2 gm. twice a week. In other cases the symptoms were mild and never incapacitating.

In a group of 35 healthy male undergraduates, who received mepacrine 0.1 gm. daily for five months and who were given the opportunity each week to report symptoms on their own initiative, no gastro-intestinal symptoms were brought to the notice of the medical officers-in-charge. This was not surprising, in view of the facts that the symptoms reported by the women were mild and that none of the men was on a régime of 0.2 gm. twice weekly.

Fifteen soldier volunteers under daily supervision reported no symptoms.

Fractional Gastric Analysis. Fractional gastric analyses were performed in 12 soldier volunteers the second day after beginning a course of mepacrine 0.1 gm. a day. The test meal was given one half-hour after the tablet. Except for a diminution in the volume of the resting juice, the results of the analyses were not significantly different from the results obtained after previous control meals.

Gastroscopy. The gastric mucosa was examined in 12 soldier volunteers the fourth day after beginning a course of mepacrine 0.1 gm. a day. The mepacrine tablet was swallowed five minutes before the introduction of the gastroscope, and in some cases it was possible to observe the tablet lying on the mucosa for as long as half an hour. In no volunteer was the appearance of the mucosa as a whole different from that seen at the control examination, but a zone of hyperaemia developed around the tablet in five to ten minutes, a small amount of thick ropy mucus accumulated, and the rugae were increased in size. This is evidence of a direct local action, but the effect was slight and not sufficient in itself to explain the symptoms which are sometimes seen.

Barium Meals. Six of the women undergraduates who had gastro-intestinal symptoms while on mepacrine submitted to examination by barium meal and radiography, and each of them presented slight abnormalities. These were hypertonicity of the stomach, pylorospasm, irritability of the small intestine, and delay at the ileo-caecal valve.

No abnormalities were found in 12 soldier volunteers who were given barium meals one hour after their third daily dose of mepacrine 0.1 gm.—the time at which symptoms were most frequently seen in the women volunteers. Six women volunteers who had been taking a suppressive dosage of mepacrine without symptoms for three to five months were also normal on examination after a barium meal.

Bacterial Flora of the Faeces. All volunteers who complained of diarrhoea submitted specimens of stool for examination. A non-lactose fermenting organism was discovered in one case; otherwise no abnormality was encountered. Duplicate quantitative examinations of the faecal flora of six women volunteers who had been receiving a suppressive dosage of mepacrine showed the relative numbers of different organisms present to be the same as in a group of eight women who were controls.

Effect of a Suppressive Dosage of Mepacrine on the Inflamed Colon. Mepacrine 0.1 gm. daily was given to two patients with chronic ulcerative colitis, who were under the care of Professor L. J. Witts, to determine whether the drug had an adverse effect on the inflamed colon. One patient was in an active phase of the disease and was having five or six loose motions daily. The other patient had been in a quiescent stage for two or three months before the experiment and was having one or two formed motions daily. Suppressive mepacrine for a fortnight did not cause any change in the symptoms or general condition, and there was no change in the bacterial flora of the stools.

THE EFFECTS OF MEPACRINE 0.2 GM. TWICE DAILY

Finally, work was undertaken on the problem presented by the large-scale vomiting and diarrhoea which occurred in North Africa in 1943 following the administration of two tablets of mepacrine on Tuesdays and Thursdays. The Consulting Physician, A.F.H.Q., Brigadier E. R. Boland, gave the following description of the clinical picture seen at that time.

'The attack generally began with nausea and vomiting accompanied by headache, chilliness, pains in the epigastrium or muscles. In milder cases there was nausea without vomiting and with abdominal discomfort, followed after an interval by a loose stool or two. When it occurred, vomiting was repeated and generally succeeded by diarrhoea, the motions being soft or loose and often frankly fluid. In a few cases diarrhoea preceded the vomiting and sometimes vomiting or abdominal pain or discomfort was a single feature. Headache and depression were very common. Initial or repeated shivering and pains in the back or legs or joints were general. The temperature was raised to 99°, 100° to 103° F., in many, especially the more seriously ill.'

Symptoms occurred most frequently after the third dose, and they developed in from three to ten hours after the dose had been given.

It seemed strange that a dose of two tablets should have caused more severe disturbances than those which occurred after a single dose of 10 tablets. Of the many explanations offered none survived examination. The suggestion that the entire episode was psychogenic in origin was rejected by clinicians on the spot. That the batches of mepacrine involved were not especially toxic was proved by examination of samples which were returned to England. No bacteriological cause was found by the pathologists of the area, and the short interval between the ingestion of the tablets and the onset of symptoms made it impossible that the cause had been some action of mepacrine on the bacterial flora of the intestine. The suggestion that mepacrine has more than the usual irritative effect on the inflamed colon was negated by our own experiments with cases of ulcerative colitis. It was decided, therefore, to put a group of volunteers on the twice-weekly régime and to study them by means of radiography and by frequent estimations of the plasma mepacrine.

Eight officers from a British General Hospital were given mepacrine 0.2 gm. at 9.00 a.m. on the 1st, 6th, 8th, 13th and 15th days, which was precisely the régime used in North Africa. On the 1st, 6th, 8th and 15th days blood was taken for plasma mepacrine estimation at 1-, 2-, 4-, 8- and 12-hour intervals after ingestion of the tablets. The officers lived on the ordinary Army diet and remained on duty except when prevented by symptoms.

Six of the volunteer-officers showed no untoward effects. The other two were severely affected on the 8th day, less affected on the 13th day and only slightly on the 15th day.

Volunteer 402. 8th Day.—9.00 a.m.: mepacrine 0.2 gm. 2.00 p.m.: nausea, epigastric discomfort and frontal headache. Pale, sweating, and thirsty. Tenderness in epigastrium and right hypochondrium. Temperature 101.6° F. 7.30 p.m.: began to vomit, and vomited frequently during the next five hours. At 7.00 p.m. a barium meal was given and examination of the gastro-intestinal tract begun. There was delay in opening of the pyloric sphincter and an excess of gastric secretion. For two hours no barium left the stomach. The normal pattern of the valvulae conniventes in the small intestine was replaced by ragged clumps of barium. The stomach remained full of secretion at four hours. The colonic outline was normal.

9th Day.—Marked general malaise; vomited once early in the morning. No abdominal tenderness. Temperature 101.4° F.

10th Day.—No complaints. Temperature 98.8° F.

13th Day.—9.00 a.m.: mepacrine 0.2 gm. 12.30 p.m.: nausea, followed by retching and vomiting which lasted for two hours; one loose motion. Detectable enlargement of the spleen. Examination by barium meal showed changes similar to those seen on the 8th day, but this time they were less marked.

15th Day.—9.00 a.m. : mepacrine 0.2 gm. 2.00 p.m. : slight nausea for one hour. Spleen no longer palpable.

Volunteer 404. 8th Day.—9.00 a.m. : mepacrine 0.2 gm. 2.30 p.m. : headache, dizziness, severe abdominal pain, onset of profuse and finally watery diarrhoea. No abnormal physical signs on examination. Temperature 101° F. 7.00 p.m. : barium meal given. There was marked hyper-secretion and some spasm of the stomach. The pylorus was open and emptying occurred at a fairly normal rate. The duodenal cap was grossly enlarged—probably a congenital abnormality. The small intestine pattern was abnormal and showed irregular fragmentation of the barium column and an increase in segmentation. The colon was spastic and there was an excess of secretion.

9th Day.—Marked general malaise and slight abdominal discomfort. Two loose bowel motions. Spleen one inch below costal margin and slightly tender. Temperature 101° F.

10th Day.—No complaints. Spleen remained enlarged. Temperature 99.6° F.

11th Day.—Spleen just palpable. Temperature 98.6° F.

13th Day.—9.00 a.m. : mepacrine 0.2 gm. 1.45 p.m. : nausea, vomiting and watery diarrhoea. Spleen one-half inch below costal margin. Temperature 98.6° F. Barium meal showed changes similar to those seen on the 8th day, but they were less marked.

15th Day.—9.00 a.m. : mepacrine 0.2 gm. 2.00 p.m. : headache, which lessened towards the evening.

17th Day.—Spleen no longer palpable.

There was no significant difference between the plasma mepacrine concentrations in the affected and in the unaffected volunteers. The peak plasma concentrations during the post-absorption period were approximately the same as on the 1st, 6th, 8th and 15th days, and varied between 35 and 45 micrograms per 1,000 ml. After a single dose of mepacrine 1.0 gm. the peak concentration is of the order of 100 micrograms per 1,000 ml. Bacteriological examination of the stools revealed no pathogenic organisms.

DISCUSSION

It is necessary before attributing symptoms to the administration of a drug to get a clear picture both of the symptoms themselves and of the findings on investigation under circumstances in which it is improbable that there are other causal factors. This is difficult to obtain in the field, where gastro-intestinal disturbances are frequent and their causes varied. Our early experiments with volunteers who took large doses of mepacrine in controlled conditions and after previous examination provided a picture with considerable definition. The syndrome which appeared in these experiments had certain precise characteristics. The vomiting was characterized by forcefulness of the first emesis, and the diarrhoea by the extreme urgency of the first bowel motion. When pain occurred it was always colicky in nature. Recovery was rapid, and in moderately affected cases considerable nausea, with vomiting or diarrhoea, was followed in a few hours by a feeling of complete well-being. There was a strict time-relation between the onset of symptoms and the ingestion of the drug, and a regular march of symptoms which towards the end of the experiment made possible a precise prognosis. The radiographic signs were equally definite. The difficulty in interpretation which arises from the variability of normal subjects was met by the results of the control examinations. The duplicate examinations of 16 subjects provided data on the day-to-day variation which occurs in the motility of the gastro-intestinal tract of healthy persons, and the large number of persons given at least one barium meal provided evidence of the variation to be expected from person to person. Each set of films taken after the administration of mepacrine was considered in relation to the control films of the same subject, and also in the light of our findings in other normal persons. In this way it was possible to form a reliable opinion concerning

the changes which could be considered due to mepacrine. These changes were gastric hypersecretion, hyperperistalsis, hypertony followed by atony, and pylorospasm; a disturbance of the motility and secretion of the small intestine, which caused irregular fragmentation of the barium stream and increased segmentation; delay at the ileo-caecal valve, and an increased rate of passage of the meal through the colon.

Are these symptoms and signs seen after the administration of mepacrine 0.1 gm. a day? They were not complained of by any of a group of 50 men receiving this dose of mepacrine over a period of five months, and neither in those men who were examined radiographically on the third day of their course nor in the women who were examined after several months were abnormalities found after barium meals. In a group of 70 women the same dose caused symptoms in seven cases, most frequently after the first few tablets. The symptoms were mild and never incapacitating, and they disappeared when the drug was continued. They were attributed to mepacrine because they disappeared when a placebo was substituted for the mepacrine tablet, and because those cases which were examined presented radiographic abnormalities similar to those found in our early experiments. Only very slight and unimportant gastro-intestinal disturbances can be attributed to mepacrine during a suppressive régime of 0.1 gm. a day, and such disturbances occur most frequently during the first week of administration and disappear when the drug is continued. Bispham (1941) arrived at the same conclusion after a study of 49,681 cases in which mepacrine had been used.

When mepacrine is given twice a week, gastro-intestinal symptoms occur more frequently, and on some régimes may be very severe. This has been noted by other observers. Loughlin *et al.* (1943) found that 0.2 gm. 'twice weekly' was followed by nausea, vomiting and diarrhoea. Missiroli (1944) found that doses of 0.3 gm. caused similar symptoms in a proportion of a rural population. In our experiments mepacrine 0.2 gm. on 1st, 4th, 8th days, etc., caused symptoms in 20 per cent. of cases. Much the most serious symptoms have followed the use of mepacrine 0.2 gm. on 1st, 6th, 8th days, etc. Any suppressive régime other than the one involving a regular daily dose of mepacrine is strongly contra-indicated.

The explanation of the very severe effects of the dosage used in North Africa in 1943 is not entirely clear, but a hypothesis can be stated. The clinical picture and the radiographic findings were the same as those found in our first experiment with volunteers who had taken very large doses of mepacrine, and it is reasonable to suppose that they were caused by mepacrine. This supposition gains strength from the failure of other suggested explanations to survive experimental testing (*cf.* above). Why did a small dose of mepacrine (0.2 gm.) cause symptoms very much more severe than were seen after a dose five times as large? It could not have been due to an accumulation of the drug, because a much larger amount can be given in daily doses for the same period without effect, and because symptoms disappeared despite continued administration. It could not have been due to the higher plasma mepacrine concentrations seen after doses of 0.2 gm., because the peak concentrations were practically as high on the 1st and 6th days when there were no symptoms, and because they were very much lower than the levels seen after a dose of 1.0 gm., when symptoms occurred but were less severe. Evidently at the time of the third dose in this régime (8th day) a proportion of subjects have a diminished tolerance of a dose of mepacrine which previously had no effect and later has no effect if the drug is continued. The spacing of the first two doses results in some subjects being less tolerant of

the drug than other subjects, and less tolerant than they themselves were at the beginning. It is probable that the catastrophe in North Africa in 1943 was due to a temporary diminution of tolerance to the drug, the unfortunate result of the particular régime which was used. Any régime with intervals between doses greater than two days will give rise to some trouble, but this particular régime causes the most severe disturbance.

SUMMARY

1. The gastro-intestinal effects of mepacrine have been investigated clinically, and by radiographic examination after a barium meal, cholecystography, gastroscopy, fractional gastric analysis, and bacteriological examination of the faeces.

2. Gastro-intestinal disturbances during a suppressive régime of mepacrine 0.1 gm. a day are unusual and are slight and unimportant.

3. A suppressive régime with intervals between doses greater than two days will cause more trouble, and such a régime should never be used.

ACKNOWLEDGEMENTS.—Great credit should be given to the 135 men and women on whom these observations were made. The co-operation and fortitude of the 25 women undergraduates and the 15 soldiers who submitted to intensive special investigation were particularly commendable.

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THE ACTION *IN VITRO* OF DIAMIDINES AND OTHER COMPOUNDS ON *LEISHMANIA DONOVANI*

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Prior to the introduction of diamidines for the treatment of leishmaniasis (Adler and Tchernomoretz, 1939; Adams and Yorke, 1939) a number of investigators had reported on the action of other remedies on the causal organisms *in vitro* (e.g., Kligler, 1924; Noguchi, 1924; Das Gupta and Dikshit, 1929). The introduction of the diamidines stimulated us to examine these compounds also in respect of their power against *Leishmania in vitro*, and to improve on techniques used by other workers for investigations of this nature. When our study was almost concluded, an account appeared of similar work with diamidines by Adler, Tchernomoretz and Ber (1945), employing different methods from ours and arriving also at somewhat different conclusions, which will be referred to later in the discussion of our own findings.

The earlier work, i.e., that prior to the introduction of the diamidines, had failed to show any marked susceptibility of *Leishmania in vitro* to compounds of proved value in the treatment of leishmaniasis. This lack of correspondence between *in vitro* and *in vivo* effects may of course be very readily explained in a number of ways, of which the three following are probably the most significant. Firstly, in the infected vertebrate it is the Leishman-Donovan bodies which are exposed to treatment, not the leptomonads of the culture-tube (and the invertebrate host), and there is every likelihood that these two forms of the parasite differ in their sensitivity to drugs. Secondly, there is the possibility that the parasites are only affected *in vivo* by the compounds in question after the latter have undergone a chemical change to an active form, as in the classical case of studies with trypanosomes, where tryparsamide itself is relatively inactive but becomes highly trypanocidal only after being reduced to the corresponding trivalent form. Thirdly, a feature common to all the earlier work is that the *in vitro* tests were carried out either at room-temperature or at 20–25° C., the optimum for artificial culture of *Leishmania*, and not in the region of 37° C., at which, perforce, drugs act *in vivo*. The importance of conducting investigations of this nature at body-temperature was stressed, and fully exploited, by Yorke and Murgatroyd (1930) in their studies of the action of drugs on pathogenic trypanosomes.

We decided to pay special attention to the third of the above considerations, i.e., to the conduct of our *in vitro* tests at 37° C., with the idea that, as in similar work with trypanosomes, maximal activity might be demonstrated at this temperature. In the event

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of leishmanicidal (or leishmanistatic) properties of a high order being elicited by our technique, we hoped that the method might have some value as a screening procedure for the selection of compounds of potential value against leishmaniasis. However, the immediate difficulty in pursuing work along these lines is that *Leishmania* parasites cannot be maintained in artificial culture at 37° C. Thus, Christophers, Shortt and Barraud (1925), investigating temperature requirements for cultivation of 28 strains of *Leishmania donovani* from Assam, in NNN medium, found the extreme range to be 16° C. to 34° C. Most strains grew unsatisfactorily, or not at all, at 30° C. or 32° C. Our first objective therefore was to attempt to acclimatize a culture of *L. donovani* to 37° C.

ATTEMPTS TO ACCLIMATIZE A CULTURE OF *L. DONOVANI* TO 37° C.

The culture medium used throughout the investigations of this paper was a clear fluid, composed of one part of fresh unheated rabbit serum mixed with two parts of 12.5 per cent. rabbit red blood-cell solution in isotonic saline. The red blood-cell solution had previously been obtained by laking one volume of defibrinated rabbit blood with six volumes of sterile distilled water, adding one volume of 6.3 per cent. sodium chloride solution, centrifuging, and discarding the deposit. We chose a medium free of agar or other gross particulate matter in order to obviate any fallacy which might arise through adsorption of the compounds tested *in vitro*.

For the acclimatization studies we used a random strain of *L. donovani* isolated from a case of Indian kala-azar in 1939, and subsequently maintained for three years in hamsters and mice and thereafter for several years in culture *in vitro*. It was found that this strain could, without any prior habituation, be continuously cultivated at a maximum temperature as high as 34° C., which is appreciably higher than the maxima reported by Christophers, Shortt and Barraud for the great majority of their strains in NNN medium. Our first attempt at acclimatizing the strain to abnormally high temperatures consisted in growing it (with subcultures every five or six days) for five months at 30–33° C., i.e., above the optimum for rich growth, but within the strain's normal capacity to grow. For the next seven months it was maintained (with subcultures every four or five days) at 35° to 36°, i.e., at temperatures above its normal capacity for growth. The steps in this experiment are shown below:

Weeks from start	Temperature (° C.)
1–6 7–20	30–31 33 } Within range of the strain's normal capacity to grow.
21–37 38–52	35 35–36 } Outside range of the strain's normal capacity to grow.

During the final period, 38–52 weeks from the start, subcultures at 36° C. were occasionally successful, but at the end of the experiment, after cultivation at relatively high temperatures for a year, it was still not possible to maintain the strain continuously at 36° C. The extent of acclimatization achieved was therefore no more than to raise by a single degree centigrade the upper level at which the strain could be continuously maintained. This method of acclimatization was evidently inadequate to achieve the desired object over any reasonable period of time; if continued for years it might perhaps

succeed—Jennings (1929) quotes an extraordinary claim by Dallinger (1887) to have acclimatized three species of free-living protozoa, over a period of seven years, to the effect that whereas originally they could not be continuously cultivated at temperatures above 26° C. they were finally capable of flourishing at 70° C.

We decided to try a second method of acclimatization, in which brief periods of exposure to excessive temperatures alternate with longer intervals at temperatures suitable for vigorous growth. A culture of the above strain was exposed to between 39° C. and 41° C. for some hours, and then returned to 25° C., at which the survivors were allowed to multiply freely. When vigorous growth was re-established in subcultures, the strain was again subjected to excessive temperatures for a brief period, as before, and then again restored to 25° C. This procedure was repeated a number of times, as follows :

Exposure	Duration (hrs.)	Temperature (° C.)
1	4	39-41
2	18	39-40
3	18	40
4	18	40-41
5	48	38-39
6	30	39-40
7	30	39-41
8	48	39-40
9	30	39-40

Immediately before the ninth exposure, the strain was tested at 37° C., but failed to grow. Any temperature-adaptation which may have been acquired must therefore have been very slight. After the ninth exposure (at 39-40° C. for 30 hours) the strain failed also to grow at 25° C., although it had previously withstood a number of similar (and even longer) exposures. Possibly its vitality had been impaired by the experience of abnormally high temperatures, as was demonstrated (though under very different experimental conditions) for a strain of *Stylonichia pustulata* by Middleton (1918), quoted by Jennings (1929).

We therefore abandoned further attempts to acclimatize the parasites to 37° C., and decided to investigate drug-action *in vitro* at the highest temperature at which rich cultures could regularly be obtained.

ACTIVITY OF DRUGS IN VITRO ON *L. DONOVANI* AT 34° C.

For these observations we chose another strain of *L. donovani*, isolated from a case of Indian kala-azar in February, 1943, and maintained thereafter in culture. It was found that, whilst this strain could, without any prior habituation, be maintained continuously, though not in florid culture, at 35° C., i.e., one degree higher than in the case of the strain referred to above, really rich growth could regularly be obtained at 34° C. We decided, therefore, to carry out our observations *in vitro* at this temperature, as being the nearest attainable towards body-temperature, for the type of work envisaged.

The method was to set up serial twofold dilutions of the drugs to be tested, in 0.45 ml. of the medium described above, in Kahn tubes covered with small glass caps. Each tube was then sown with 0.025 ml. of a suspension of flagellates prepared as follows.

A six- to eight-day 24° C. culture was lightly centrifuged, to throw down the numerous clumps of flagellates invariably present, which were then discarded. The supernatant fluid was then stirred, to obtain a uniform suspension of the remaining isolated individuals, whose concentration was determined by means of a haemocytometer. Either by further centrifugation or by dilution, as might be required, the concentration of the suspension was finally adjusted to 1,400–1,700 per c.mm., and it was then ready for sowing, as described above, 0.025 c.mm. into each tube of 0.45 ml. of drug solution in culture medium. The tubes were then incubated at 34° C. for five days (the life of control cultures being about seven days), when a drop from each tube was examined by microscope to determine the lowest concentration at which parasites were not to be found. The end-point was usually quite definite.

The enhancement of drug-action *in vitro* at 34° C. compared with 24° C. is well shown in Table I, which records the leishmanicidal* titres of three diamidines as observed in a five-day experiment at these two temperatures. It may be seen that these compounds

TABLE I
Showing activity of three aromatic diamidines on *Leishmania donovani* *in vitro* at 24° C. and 34° C. respectively

Compound	Leishmanicidal titre	
	24° C.	34° C.
4 : 4'-diamidino diphenyl ether (phenamidine) ...	50,000	500,000
4 : 4'-diamidino diphenoxy pentane (pentamidine)	250,000	4,000,000
4 : 4'-diamidino stilbene (stilbamidine)	125,000	2,000,000

were 10–16 times more active at 34° than at 24° C., stilbamidine and pentamidine being effective at concentrations as low as one in two million and one in four million respectively at 34° C., the temperature chosen for the main series of tests set out below.

Table II shows the results of *in vitro* tests with a considerable number of compounds. Many of these (as indicated on the table) have already been tested for their action on leishmaniasis in hamsters, and it is therefore possible to form some idea of the degree of correlation between their *in vitro* and their *in vivo* effects. The evaluation of their action *in vivo*, as reported by those who have carried out that investigation, has been approximately represented by us on the table by the symbols ++ (for very active); + (active); ± (slightly or doubtfully active); and — (insignificantly active or inactive). The following are broad conclusions to be drawn from the table, with a discussion of some of the points arising.

1. The only compounds showing considerable activity *in vitro*, i.e., to a titre of one million or more, were some (though not all) of the diamidines.
2. Among diamidines and closely related compounds examined both *in vitro* and

* Throughout this paper the action of the diamidines under our experimental conditions is described as leishmanicidal, without prejudice as to whether leishmanistatic action would not be a more correct formal interpretation. No doubt both modes of action operate to some extent, but we have made no attempt to determine which predominates.

TABLE II

Showing activity of various compounds on *Leishmania donovani* in vitro at 34° C. and in hamsters

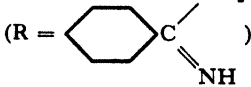

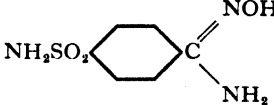
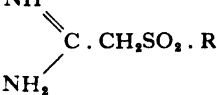


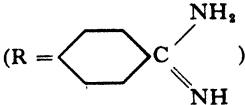
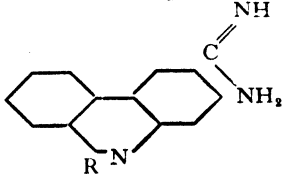

Compound*		Formula 	Leish- manicidal titre in vitro	Activity in hamsters†	
1	4 : 4'-Diamidino diphenyl ether (phenami- dine)	R . O . R	250,000	—	Adler and Tcherno- moretz (1939, 1942)
2	4 : 4'-Diamidino phenyl benzyl ether ...	RCH ₂ . O . R	250,000	—	
3	4 : 4'-Diamidino diphenoxy ethane ...	RO(CH ₂) ₂ OR	250,000	±	
4	4 : 4'-Diamidino diphenoxy propane (prop- amidine)	RO(CH ₂) ₃ OR	2,000,000	++	
5	4 : 4'-Diamidino diphenoxy pentane (pent- amidine)	RO(CH ₂) ₅ OR	4,000,000	+	
6	4 : 4'-Diamidino diphenyl ethane ...	R(CH ₂) ₂ R	250,000	—	
7	4 : 4'-Diamidino stilbene (stilbamidine) ...	R . CH : CH . R	2,000,000	++	
8	4 : 4'-Diamidino monomethyl stilbene ...	R . CCH ₃ : CH . R	1,000,000	+	Fulton (1944)
9	4 : 4'-Diamidino dimethyl stilbene ...	R . CCH ₃ : CCH ₃ . R	4,000,000	+	
10	4 : 4'-Diamidino 2-hydroxy stilbene ...	R . CH : CH . R	1,000,000	++	
11	4 : 4'-Diamidino tolane ...	 R . C : C . R	500,000	±	
12	4 : 4'-Diamidino diphenyl hexane ...	R(CH ₂) ₆ R	2,000,000	—	
13	4 : 4'-Diamidino diphenyl urea ...	R . NH . CO . NH . R	500,000	±	
14	4 : 4'-Diamidino diphenyl sulphone ...	R . SO ₂ . R	4,000	—	
15	4-Sulphonamidobenzamidine ...	NH ₂ SO ₂ . R	< 500	—	Fulton (personal communi- cation)
16	4-Sulphonamidobenzamidoxime ...		< 500	—	
17	4-Methylsulphonylbzenzamidine ...	CH ₃ SO ₂ . R	< 500	—	
18	4-Amidinomethylsulphonylbzenzamidine ...		500	—	
19	4-Methylsulphonylbenzylamine ...	CH ₃ SO ₂  CH ₂ NH ₂	8,000	—	
20	4-Sulphonamidobenzylamine (marfanil) ...	NH ₂ SO ₂  CH ₂ NH ₂	< 16,000	—	
21	4 : 4'-Diamidino dibenzyl ether ...	RCH ₂ . O . CH ₂ R	62,500		
22	4 : 4'-Diamidino diphenoxy hexane ...	RO(CH ₂) ₆ OR	2,000,000		
23	4 : 4'-Diamidino diphenoxy heptane ...	RO(CH ₂) ₇ OR	2,000,000		
24	2 : 2'-Dibromo-4 : 4'-diamidino diphenoxy propane	Br RO(CH ₂) ₃ OR Br	500,000		
25	2 : 2'-Di-iodo-4 : 4'-diamidino diphenoxy propane	I RO (CH ₂) ₃ ORI	1,000,000		
26	2 : 6-Di-iodo-4 : 4'-diamidino diphenoxy propane	I . IRO(CH ₂) ₃ OR	125,000		
27	2 : 2'-Dibromo-4 : 4'-diamidino diphenoxy pentane	Br RO(CH ₂) ₅ OR Br	500,000		
28	2-Bromo-4 : 4'-diamidino diphenoxy pen- tane	Br RO(CH ₂) ₅ OR	500,000		
29	4 : 4'-Diamidino-αβ-dibromo-dibenzyl ...	R CH Br . CH Br R	1,000,000		
30	4 : 4'-Diamidino-γδ-diphenylhexane ...	R CH Et . CH Et R	62,500		
31	4 : 4'-Diamidino diphenylamine ...	R . NH . R	2,000,000		

TABLE II—continued

Compound*		Formula 	Leish- manicidal titre <i>in vitro</i>
32	4 : 4'-Di-N-ethylamidinostilbene ...	EtRCH : CHREt (Et substitutions in the amidino groups)	1,000,000
33	3-Amidino-9-(<i>p</i> -amidinophenyl)-phenanthridine		500,000
34	Antimonyl potassium tartrate ...	(CH . OH . COO) ₂ K . SbO	40,000
35	N-Glucoside of sodium <i>p</i> -aminophenylstibonate (neostam)	NaHO ₂ Sb  . C ₆ H ₁₁ O ₅	1,600
36	Mepacrine Proflavine Surfen C 3349 (see Curd, Davey and Rose, 1946)		400,000
37			400,000
38			<25,000
39			100,000
40	Penicillin		Min. lethal conc. 100 units/ml.

* Diamidines were used in the form of dihydrochlorides, di-isethionates or dilactates. Titres recorded represent concentrations of the base.

† — = Inactive. ± = Doubtfully or slightly active. + = Active. ++ = Very active.

in vivo (Nos. 1–20) there was a fair correlation of activity under these two conditions. In general, compounds with a titre of one million or more were effective also *in vivo*, whilst those with a lower titre were ineffective or only feebly active *in vivo*. The one outstanding exception to this generalization is the case of 4 : 4'-diamidino diphenyl hexane (No. 12 of Table II), which was found to have a titre of two million, but which Fulton (1944) found to be ineffective *in vivo*. The broad conclusion seems nevertheless justified that the *in vitro* technique described should be of some value as a screening procedure, in this particular chemical group, for compounds effective against leishmaniasis.

3. Since the flagellate form of *L. donovani* is so highly susceptible *in vitro* to those diamidines which have been shown to be effective against leishmaniasis, it is not unlikely that the Leishman-Donovan body is likewise sensitive to the direct action of those compounds, whose mode of action against leishmaniasis is accordingly partly to be explained on this basis. This is supported by the recent claim of Reed and Anderson (1945) to have demonstrated a degree of activity of stilbamidine on Leishman-Donovan bodies *in vitro* commensurate with its action *in vivo*.

4. Antimonials of proved efficacy against leishmaniasis, i.e., tartar emetic and neostam, showed an extremely low degree of activity *in vitro*. This confirms, for experiments conducted nearer to body-temperature, the conclusions of previous workers in experiments carried out at room-temperature or at 20–25° C. The mode of action of antimonials

against leishmaniasis remains obscure. It must, of course, be borne in mind that, as pointed out above, there is no sound *a priori* reason for expecting the reactivity of the flagellate form to be any index to the reactivity of the Leishman-Donovan body. It does seem, from our experience with the diamidines, that where a compound is exceptionally active against the flagellate form we may anticipate an unusually high grade of effectiveness against the Leishman-Donovan body; but where a compound is relatively inactive against the flagellate form there is evidently no ground for expecting, therefore, that it would likewise be lacking in activity against the Leishman-Donovan body. Apart from considerations of the difference between leptomonads and Leishman-Donovan bodies there is, of course, the possibility with antimonials that, as has been shown for arsenicals and trypanosomes, therapeutically efficacious pentavalent compounds are relatively inactive *in vitro* and only display their powers on reduction to the corresponding trivalent form. It is true that the trivalent tartar emetic, which exhibits a high degree of activity against trypanosomes *in vitro* (Yorke and Murgatroyd, 1930), is only slightly active against Leishmania flagellates. Nevertheless, in our *in vitro* tests this substance was found to be some 25 times as active as the pentavalent neostam (titre of 40,000, as against 1,600). This suggests that pentavalent antimonials are in fact reduced to the trivalent state as a first step towards exercising any effect on leishmaniasis; such a valency-change has been shown by Goodwin and Page (1943) to occur *in vivo* after injection of sodium antimony gluconate, and *in vitro* on incubation of this compound in the presence of living tissues, blood, or preparations of liver or kidney. It is, of course, not unlikely that it is not the trivalent analogue of a therapeutic pentavalent compound which is actually responsible for activity, but that some further change in the molecule is necessary.

5. Claims have been made for mepacrine and for various acridine antiseptics of the type of proflavine, structurally related to mepacrine, in the treatment of cutaneous leishmaniasis (Flarer, 1938; Marchionini, 1941; and others). The table shows that these compounds are moderately effective *in vitro* against *L. donovani*, the parasite of visceral leishmaniasis. The question naturally arises whether other compounds exhibiting the same degree of activity *in vitro* against *L. donovani* may not likewise exercise some influence on cutaneous leishmaniasis.

6. Miscellaneous compounds found to possess insignificant activity against Leishmania *in vitro* were (a) penicillin (as has since been reported by Fulton, 1945), (b) 3349, one of a series of compounds recently shown to exhibit antimalarial action (Curd, Davey and Rose, 1945), and (c) Surfen C, a German aminoquinoline preparation of some value against *Trypanosoma congolense* infections.

RELATIONSHIP BETWEEN *IN VITRO* LEISHMANICIDAL ACTIVITY AND CHEMICAL CONSTITUTION OF DIAMIDINES AND RELATED COMPOUNDS

It may now be profitable to examine in greater detail the results obtained with diamidines and closely related compounds, and to discover the relationships between chemical constitution and *in vitro* leishmanicidal activity within the group. The following conclusions seem justified, and will be more clearly appreciated if read in conjunction with Table III, in which the compounds are arranged in their respective chemical categories, as well as in the order of their *in vitro* activity. To facilitate reference, the paragraph-

Reference to text*	para.	4,000,000	2,000,000	1,000,000	500,000	250,000	125,000	62,500	<16,000	Reference to text*	para.
1a)		RO(CH ₂) ₃ OR	RO(CH ₂) ₃ OR			RO(CH ₂) ₂ OR				3	
			RO(CH ₂) ₆ OR								
			RO(CH ₂) ₇ OR								
			R(CH ₂) ₆ R			R(CH ₂) ₂ R					
1b)				RCHBr·CHBr·R	RC:CR	ROR		RCH ₂ OCH ₂ R	RSO ₂ R	3	
		RC(CH ₃):C(CH ₃)R	RCH:CHR	OH·RCH:CHR	RNHCONHR	RCH ₂ OR		RCH(C ₂ H ₅)CH(C ₂ H ₅)R			
1c)				RC(CH ₃):CHR						4	
				EtRCH:CHREt							
1d)			R·NH ₂ ·R								
2				IRO(CH ₂) ₃ ORI	BrRO(CH ₂) ₅ ORBr		1IRO(CH ₂) ₅ OR			5	
					BrRO(CH ₂) ₅ ORBr						
					BrRO(CH ₂) ₅ OR						

Highly active

Less active,³ and relatively inactive

numbers below are repeated on the first and last columns of the table, in order to indicate which sections of the table are referred to by each paragraph.

1. The most active compounds appear in the following categories ($R = \text{Cyclohexyl-C}(\text{NH}_2)_2$):—

- (a) $R \text{ O}(\text{CH}_2)_n \text{ O R}$, and $R(\text{CH}_2)_n \text{ R}$, where n is, in both groups, more than 2.
- (b) The $\alpha\beta$ -dibromo derivative of the latter group, n being 2, i.e., $R \text{ CHBr} \cdot \text{CHBr R}$.
- (c) $R \text{ CH} : \text{CH R}$, and derivatives (2-hydroxy, α -methyl, $\alpha\beta$ -dimethyl, and di- N -ethyl).
- (d) $R \cdot \text{NH} \cdot \text{R}$.

2. Activity of $R \text{ O}(\text{CH}_2)_n \text{ O R}$ seemed generally to be somewhat impaired by introduction of a halogen into one or both benzene rings. Activity was considerably impaired in the one compound tested in which two halogen atoms (iodine) were introduced into one of the rings.

3. With the exception of (c) and (d) above, all diamidines with short-chain linkages between the aryl nuclei show only moderate or slight activity. These are, in four groups, in descending order of activity:

- (a) $R \text{ C} : \text{CR}$ and $R \text{ NH} \cdot \text{CO} \cdot \text{NH R}$.
- (b) $\text{RO}(\text{CH}_2)_n \text{ OR}$, $R(\text{CH}_2)_n \text{ R}$, ROR , and RCH_2OR .
- (c) $R \text{ CH}_2\text{OCH}_2\text{R}$ and $\text{RCHEt} \cdot \text{CHEtR}$.
- (d) $R \text{ SO}_2\text{R}$.

4. Moderate activity was shown by a phenanthridine compound containing an amidine group in the 3-position and a benzamidine group in the 9-position.

5. Insignificant activity was shown by miscellaneous sulphonyl and sulphonamido-benzene compounds, containing p -amidine, aminomethyl, and amidoxime groupings, some of which compounds are known to be active against experimental typhus (Andrewes *et al.*, 1944), gas-gangrene (Evans *et al.*, 1944), and tetanus (Evans *et al.*, 1945).

This is a suitable point at which to consider the similar work of Adler, Tchernomoretz and Ber (1945). These workers examined *in vitro* seven diamidines, whose action on leishmaniasis in hamsters they had already described (see our Table II). Their observations were carried out (a) at 24° C. and (b) and (c) at 37° C. for 24 and 48 hours respectively, with subsequent transfer to 24° C. The medium used was Locke-serum-agar. Our own technique seems preferable, since Adler and his colleagues found that the end-point, i.e., the minimum concentration in which living flagellates were no longer found, was not very sharp. They therefore resorted, for their main conclusions, to the somewhat laborious procedure of determining for each compound the maximum concentration which permits 90–100 per cent. growth, in relation to the growth observed in control cultures. They concluded that the degree of correlation between results *in vitro* and *in vivo* was insufficient for the *in vitro* technique to be regarded as a hopeful means of selecting compounds for trial *in vivo*. This conclusion was founded largely on the fact that, although the four compounds least effective *in vivo* proved also to be the least effective *in vitro*, the order of effectiveness *in vivo* of the remaining three compounds tested, stilbamidine, pentamidine and propamidine, did not tally with the order of their activity *in vitro*. Thus, although it had been found that stilbamidine was more effective than the other two compounds *in vivo*, it was considerably less effective *in vitro*. We, however, have found that stilbamidine exhibits a very high order of *in vitro* activity, in a class with that shown by pentamidine and propamidine, and we feel justified in the general conclusion, stated above, that our *in vitro* technique would have some value as a screening procedure, in this particular group, for compounds effective against leishmaniasis.

In searching for an explanation for the failure of stilbamidine to exhibit a high degree of activity *in vitro* in the experiments of Adler and his co-workers, two points are worth considering. Firstly, it is now well established that solutions of stilbamidine, unlike

TABLE IV

Relationship of chemical constitution of diamidines to (a) trypanocidal activity *in vivo* and (b) leishmanicidal activity *in vitro*

Chemical category		Trypanocidal activity <i>in vivo</i> (Ashley <i>et al.</i> , 1942)*	Leishmanicidal activity <i>in vitro</i> (present experiments)
Mononuclear diamidines		Little or none.	Only one compound examined (No. 18); showed negligible activity, though this may be due to SO ₂ group in the linkage.
Binuclear diamidines with :	Unsaturated hydrocarbon linkages	High activity shown by two carbon linkage RCX : CXR (X = H or CH ₃).	High activity shown by two carbon linkage RCX : CXR (X = H or CH ₃).
	Saturated hydrocarbon linkages	Marked activity shown by both two and six carbon linkages.	Low activity with two carbon chain, high activity with six.
Replacement of one CH ₃ group by :	O	Slightly enhanced activity.	No evidence of enhanced activity, since titre of R . CH ₂ OR is no higher than that of R (CH ₂) ₂ R. Indeed, a suggestion of lowered activity, since titre of R CH ₂ OCH ₂ R very low, but corresponding R (CH ₂) ₃ R not examined.
	NH	Increased activity.	Probably increased activity, since R . NH . R showed high titre, but corresponding R . CH ₂ . R not examined.
	S or SO ₂	Activity practically disappeared.	R . SO ₂ R inactive.
Chain with one ether linkage		Homology of this series shows no marked influence on activity. (No compound examined with chain of more than two carbon atoms.)	Inactive or slightly active. R CH ₂ OCH ₂ R appreciably less active than R CH ₂ OR. (No compound examined with chain of more than two carbon atoms.)
Chain with two ether linkages		Maximum activity with chains of three and five carbon atoms, then diminishing, up to 10-carbon chain.	Considerable activity with chains of three to seven carbon atoms. Longer chains not examined.
Replacement of one CH ₃ by CH(OH) or by CO		Diminished activity.	No data.
Substitution in the amidine group		Substitution of one hydrogen of the amidine group by Me or Et does not seriously impair activity.	Activity not seriously impaired on substitution by Et.
Halogenation of benzene ring or rings (compounds with two ether linkages only)		Diminished activity.	Diminished activity.
Variation in position of the amidine groups		3' : 4 diamidines differ little from corresponding 4 : 4' compounds, but 3 : 3' compounds less active.	No data.
Monoamidines of similar structure to some active diamidines		Two amidine groups needed for activity.	No data.
Compounds in which one or both amidine groups are aliphatic in character		Less active than those with corresponding length of chain in which both amidine groups are aromatic.	No data.

* The conclusions in this column do not agree in all respects with those originally published by Ashley *et al.* They have been somewhat amplified and revised by these workers in the light of their further observations, and we are obliged to Dr. Ewins for sending us the revised conclusions.

those of pentamidine and propamidine, are very unstable both chemically and in regard to their trypanocidal activity on exposure to daylight. This instability is likely to apply also to leishmanicidal activity, and the question arises whether the manipulations in the experiments of Adler and his co-workers did not allow this factor to operate. Secondly, unlike ourselves, they used a medium incorporating agar. It may be that stilbamidine is adsorbed to this substance to a degree which would reduce its availability for action on the flagellates present in the medium. There are conflicting views on the extent to which stilbamidine is adsorbed to red blood-cells (Henry and Grindley, 1942; Fulton and Goodwin, 1945).

COMPARISON BETWEEN THE RELATIONSHIP OF CHEMICAL CONSTITUTION OF AROMATIC DIAMIDINES TO (A) TRYPANOCIDAL ACTIVITY *IN VIVO* AND (B) LEISHMANICIDAL ACTIVITY *IN VITRO*

Ewins and his colleagues have described the relationship of chemical constitution to therapeutic activity in *T. equiperdum* infections of mice (Ashley *et al.*, 1942). Generalizations concluded by these authors were necessarily tentative, being based, in some of the chemical categories, on a rather limited number of compounds. For the same reason, our own generalizations on the relationship of chemical constitution to leishmanicidal activity *in vitro* must also be regarded as no more than tentative. With this as a reservation, it is nevertheless of interest to compare the conclusions of Ewins and his colleagues, in respect of trypanosomes *in vivo*, with our own in respect of *Leishmania* parasites *in vitro*. The comparison is set forth in Table IV above, where Ewins and his co-workers' conclusions (somewhat amplified and revised in the light of their subsequent work) are placed alongside our own, in regard to each chemical category; the interesting fact emerges that, in most of these categories, there is a fair degree of parallelism between the two sets of conclusions.

The over-all conclusion of Ewins and his colleagues is that the most active trypanocidal compounds are of the type $\text{NH}_2\text{C}(:\text{NH})\cdot\text{C}_6\text{H}_4\cdot\text{X}\cdot\text{C}_6\text{H}_4\cdot\text{C}(:\text{NH})\cdot\text{NH}_2$, in which X is a simple aliphatic chain in which one or more of the CH_2 groups are replaced by oxygen, or is an ethylenic linkage as in the stilbenes. Our general conclusions in regard to leishmanicidal activity *in vitro* lay no emphasis on the importance of replacement of one or more of the CH_2 groups by oxygen (although this might be established in a more comprehensive investigation), and would include statements that (a) substitution of methyl groups in an ethylenic linkage is apparently not detrimental to activity (see compounds 8 and 9, Table II), and (b) halogen substitution may be advantageous in an alkylene linkage (compare compound 29 with 6, Table II), but not in one or both benzene rings (compare compounds 24-28 with 4 and 5).

SUMMARY

1. A technique is described for measuring the activity *in vitro* of chemical compounds on *Leishmania donovani*. The medium used was free from agar, blood-cells or other gross particulate matter, in order to minimize fallacies due to any possible adsorption of compounds on the constituents of the medium.

2. It was regarded as desirable for the tests to be carried out at body-temperature, rather than at 20-25° C., the temperature at which *Leishmania* is generally cultured. With this in view, attempts were made to acclimatize cultures to growth at 37° C., but without

success. The drug-tests were therefore carried out at 34° C., the highest temperature at which satisfactory cultures of one of our strains could, without any prior habituation, regularly be obtained.

3. A total of 40 compounds, mostly aromatic diamidines or closely related substances, were tested. The only compounds showing considerable activity *in vitro* were some (though not all) of the diamidines (see also Adler *et al.*, 1945). Antimonials of known efficacy against leishmaniasis were relatively inactive.

4. Among diamidines and closely related compounds, there was a fair correlation between activity *in vitro* (our tests) and *in vivo* (tested by others). In general, compounds with a titre *in vitro* of one million or more were effective also *in vivo*, whilst those of a lower titre showed little or no activity *in vivo*. Our *in vitro* technique would therefore be of some value as a screening procedure, in this particular chemical group, for compounds effective against leishmaniasis.

5. A study has been made of the relationship between chemical constitution of diamidines (and closely related compounds) and their leishmanicidal activity *in vitro* (p. 96 and Table III). This has been compared with a similar study by Ewins and his colleagues (Ashley *et al.*, 1942) of the relationship between chemical constitution and trypanocidal activity *in vivo* (p. 98 and Table IV).

ACKNOWLEDGEMENTS.—We are obliged to Dr. A. J. Ewins, F.R.S., of Messrs. May and Baker Limited, for supply of most of the compounds tested.

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STUDIES ON SYNTHETIC ANTIMALARIAL DRUGS

XV.—HYDROLYTIC DETERMINATION OF PALUDRINE

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INTRODUCTION

An earlier paper in this series (Spinks and Tottey, 1945*b*; cf. 1945*a*) described a method of determining Paludrine by hydrolysis to *p*-chloroaniline, followed by diazotization and coupling to give an azo dye. The method has been in daily use for nearly 12 months in the laboratories of Imperial Chemical Industries Limited and at the Liverpool School of Tropical Medicine, and the experience gained suggested an amplification of the description previously given, particularly as regards the steps that were found to require special precautions. Further experimental work has also been carried out, and, as a result, the method has been modified, the duration of hydrolysis being reduced from twelve to four hours. The latter modification has been confirmed by Professor Maegraith, to whom we are also indebted for many helpful suggestions.

THE METHOD

APPARATUS

30 and 60 ml. narrow-necked, glass-stoppered bottles.

Test-tubes.

Glass-stoppered centrifuge-tubes, 20 ml. ; or

Rubber-stoppered centrifuge-tubes, 20 ml.

2 ml. ampoules, constricted.

Tubes graduated at 2 ml.

Autoclave with pressure-gauge (e.g., a Pentecon household pressure-cooker).

Photoelectric or visual colorimeter.

Pipettes, burettes, etc.

Glass apparatus is well washed in nitric-chromic acid mixture, and then at least six times in tap-water and twice in distilled water, after which it is dried in the oven. It should be kept covered until used. Apparatus containing biological material is cleaned in alkali-soap mixture before using this procedure.

REAGENTS

40 per cent. aqueous sodium hydroxide.

Benzene containing 2 per cent. of ethanol.

N/4 hydrochloric acid. ,

5 per cent. sodium nitrite.

0.1 per cent. sodium nitrite.

1 per cent. N- β -sulphatoethyl-*m*-toluidine.

30 per cent. sodium acetate.

Concentrated hydrochloric acid.

Standard Paludrine solution in N/4 hydrochloric acid, 10 mgm./100 ml.

Analar chemicals must be employed where available. Analar benzene is further purified by shaking twice with N/4 hydrochloric acid and clarified by filtration through filter-paper. If Analar benzene is unobtainable, the best quality available is shaken twice with concentrated sulphuric acid, twice with distilled water, and filtered. The quality of the benzene used should be frequently checked by 'water blank' analyses (see below). Ethanol is purified by distillation. 0.1 per cent. sodium nitrite is prepared daily by diluting the stock 5 per cent. solution, which may be kept for six months without serious deterioration. N- β -sulphatoethyl-*m*-toluidine should be quite colourless; discoloured samples may be purified by crystallization from hot water after charcoal treatment. It becomes discoloured in daylight and must be kept in the dark. The 1 per cent. solution is best prepared weekly, and should be kept in an amber-coloured, glass-stoppered bottle. Paludrine standard solutions are prepared from pure anhydrous base (rosettes from benzene, melting-point 129.5°–130° C., corrected), and should be made up freshly every two weeks and stored in the refrigerator.

ANALYSIS OF PLASMA

Plasma must be prepared using precautions against cell contamination. Whole blood is centrifuged, immediately after withdrawal, at 1,500 revolutions per minute for 30 minutes, and the plasma is carefully removed and recentrifuged for 20 minutes at 1,500 revolutions per minute. It should be crystal clear and must contain no haemoglobin, as concentrations in red cells are much higher than those in plasma. It may be kept in the refrigerator for periods of at least a week without apparent alteration in Paludrine content. The plasma (5–10 ml.) is pipetted into a 30 ml. glass-stoppered bottle, and treated with one quarter of its volume of 40 per cent. sodium hydroxide. The bottle is then warmed at 50° for 30 minutes, with occasional shaking, and allowed to cool to room-temperature. If benzene is added before cooling occurs, emulsions may form on shaking. Benzene-ethanol (10 ml.) is then added from a burette, and the bottle is vigorously shaken for five minutes, preferably on a mechanical shaker. The speed and manner of shaking greatly influence emulsion formation. It will be found best to hold or fix the bottle vertically and to shake in the horizontal plane. Emulsions then rarely form with plasma, but may do so with whole blood. The contents of the bottle are transferred to a stoppered test-tube, which is centrifuged at 4,000 revolutions per minute for 10 minutes. The benzene should then have separated completely. If an emulsion has not been resolved, the upper gelatinous layer is stirred with a glass rod and the tube is recentrifuged. If this treatment fails the tube is placed in the refrigerator until the benzene has frozen. Recentrifugation after warming to room-temperature then resolves the worst emulsions. As large an aliquot as possible of the upper layer is withdrawn by means of a 10 ml. pipette graduated in 0.1 ml. divisions, suction being by vacuum through a three-way tap, permitting complete closure and entry of air. Great care must be taken that no trace of the lower layer is removed; the final

solution may otherwise be yellow, instead of colourless or pink. The volume is noted and the benzene delivered into a glass-stoppered centrifuge-tube. If glass-stoppered centrifuge-tubes are not available rubber stoppers may be used, but these have been the most frequent sources of error so far discovered. Immediately after use, they must be well washed in distilled water, and kept until required under distilled water. They are then boiled in N/4 hydrochloric acid for 10 minutes and carefully dried with clean filter-paper. Even after observing these precautions, rubber bungs have caused trouble occasionally, and an old batch should be discarded as soon as 'water blank' analyses consistently give colours. N/4 hydrochloric acid (1.2 ml.) is added to the benzene, and the tube is stoppered and shaken vigorously for two minutes. The stopper is removed and the tube centrifuged for five minutes at 1,000 revolutions per minute. The lower layer is removed as completely as possible by means of a pipette drawn out to a capillary and provided with a rubber teat to facilitate suction. Inclusion of traces of the benzene layer should be avoided, if possible, to reduce the likelihood of an ampoule bursting in the autoclave; but this rarely occurs even when several drops of benzene are included, and the latter does not affect the subsequent analysis. The acid is transferred to an ampoule, which is then carefully sealed. The smallest leak permits escape of *p*-chloroaniline and should be avoided in the usual way by allowing the ampoule to seal completely *before* drawing out. The ampoule is autoclaved for four hours at 20–25 lb. pressure and allowed to cool. A 1 ml. aliquot is withdrawn into a tube graduated at 2 ml., 0.1 per cent. sodium nitrite (0.1 ml.) is added, and the tube is shaken. After 10 minutes 1 per cent. N- β -sulphatoethyl-*m*-toluidine (0.2 ml.) is added, followed by 30 per cent. sodium acetate (0.4 ml.), the tube being shaken after each addition. The yellow colour of the azo dye is changed to red after 10 minutes by making up to volume with concentrated hydrochloric acid. The colour obtained may be matched against that of a standard in either a photoelectric or a visual colorimeter. In either case, it is best to autoclave a series of standards along with the unknown. 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 ml. volumes of the 10 mgm./100 ml. standard are made up to 10 ml. with N/4 hydrochloric acid. These diluted standards contain 0, 1, 2, 3, 5, 7 and 10 μ gm. of Paludrine base/ml. They are autoclaved along with the unknowns and 1 ml. aliquots are similarly developed. If a photoelectric colorimeter is available, a standard curve is constructed using a filter with maximum transmission from 500 to 510 $m\mu$. From such a curve (of galvanometer reading against concentration of Paludrine in μ gm./ml.) the concentration in the final unknown aliquot is read off as y μ gm./ml. Then the concentration in the original plasma sample (c) is given by the formula

$$c = \frac{y \times 1.2 \times 10}{z \times v} \mu\text{gm./ml. (mgm./l.)}$$

where z is the aliquot of benzene, and v the volume of the plasma in ml. If a visual colorimeter is used, the standard most closely matching the unknown is chosen and the unknown read against it. The concentration, y μ gm./ml. in the final acid aliquot, is then given by the formula

$$y = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times s$$

where s is the concentration of the standard in μ gm./ml. Samples should be read within an hour of preparation, since a yellow colour, probably due to nitroso derivatives, slowly develops.

Normal plasma gives no colour. In order to check purity of reagents and cleanness of apparatus, it is desirable to analyse water, and water to which Paludrine has been added, at frequent intervals. 5 ml. volumes of aqueous standards containing 0, 0.2 and 1.0 $\mu\text{gm.}$ of Paludrine/ml. are analysed exactly as plasma. If the water blank is faintly coloured, the source of contamination must be sought. The most likely sources, apart from glass apparatus, are rubber bungs, benzene-ethanol and N/4 hydrochloric acid, in that order. If the usual precautions have been taken to avoid pipette contamination, the other reagents should never need attention.

ANALYSIS OF BLOOD

Blood (1–7.5 ml.) is pipetted into a 30 or 60 ml. glass-stoppered bottle, diluted with an equal volume of water, and treated with half its volume of 40 per cent. sodium hydroxide. After warming at 50° C. for 30 minutes and cooling, benzene-ethanol is added from a burette in a volume depending on that of the blood—that is, for 1–3, 4, 5 and 7.5 ml. of blood 10, 12, 15 and 20 ml. of benzene-ethanol respectively. Subsequent steps in the analysis are as described for plasma. Normal blood may show some colour in the final stage, equivalent usually to from 0 to 100 $\mu\text{gm.}$ of Paludrine/litre. Blood taken before administration of Paludrine should therefore be analysed along with the unknown samples, and the blank subtracted in the usual way.

Sensitivity. The method described above is adequate for the determination of Paludrine in plasma and blood (5–10 ml.) down to concentrations of 50 $\mu\text{gm.}/\text{l.}$ When lower concentrations are to be determined the following modified coupling procedure is used. 1 ml. of hydrolysate is transferred to a tube graduated at 1.7 ml., and 0.1 per cent. nitrite (0.1 ml.) is added. After 10 minutes 1 per cent. N- β -sulphatoethyl-*m*-toluidine (0.1 ml.) and 30 per cent. sodium acetate (0.3 ml.) are added, and a further 10 minutes is allowed for coupling. The volume is then made up to the mark with concentrated hydrochloric acid. The unknown is compared with a series of similarly autoclaved and developed standards containing 0, 0.1, 0.2, 0.3, 0.5, 1.0 and 2.0 $\mu\text{gm.}$ of Paludrine/ml. of N/4 hydrochloric acid. Using this modification with a Spekker absorptiometer carrying 3 cm. cells of 1.5 ml. capacity, the limit of determination is 0.05–0.1 $\mu\text{gm.}$, equivalent to 7–14 $\mu\text{gm.}$ of Paludrine/l. in 10 ml. of plasma. The 3 cm. absorptiometer cells were made to our design by Tintometer Limited.

ANALYSIS OF URINE

The concentration of Paludrine in urine from patients receiving 50–500 mgm. twice daily usually falls within the range of 10–400 mgm./l. Experience will indicate the most suitable volume of urine to use. 0.2–1.0 ml. is pipetted into a 30 ml. glass-stoppered bottle and diluted with 2 ml. of water and 2 ml. of 40 per cent. sodium hydroxide. Extraction with 10 ml. of benzene-ethanol is carried out without first heating the bottle. 2 ml. of N/4 hydrochloric acid is used in the final extraction, and, after autoclaving, a volume depending on the expected concentration of Paludrine is taken for colour development. Until experience suggests a suitable volume, 1 ml. may be used and the excess retained for dilution in N/4 hydrochloric acid if the colour developed is too strong to read. Dilution of the final solution is unsatisfactory, probably owing to an effect of inorganic solute concentration on the electrolytic dissociation of the dye. Normal urine gives no colour. If

urine must be stored before analysis, toluene is added as a preservative. No loss of Paludrine occurs on shaking urine with toluene.

ANALYSIS OF FAECES

A suitable sample (5–10 gm.) is dispersed in distilled water on a ball-mill or in a pestle and mortar. The volume to which the dispersion is adjusted will be indicated by experience; 50 ml. is usually suitable. Paludrine is strongly adsorbed on faecal solids, and care must be taken to shake the dispersion well before removing the aliquot (5 ml.). 40 per cent. sodium hydroxide (2.5 ml.) is added, and the mixture is heated at 50° for 30 minutes with frequent shaking. Subsequent steps in the analysis are as described for urine. Normal faeces give a low blank, which can be neglected.

ANALYSIS OF BILE

Concentrations of Paludrine in rabbit bile, following the administration of 10–100 mgm./kgm., are not usually greater than 10 mgm./l. Bile (1–5 ml.) is analysed exactly as blood. Normal bile usually gives no colour.

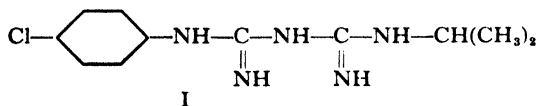
ANALYSIS OF TISSUES

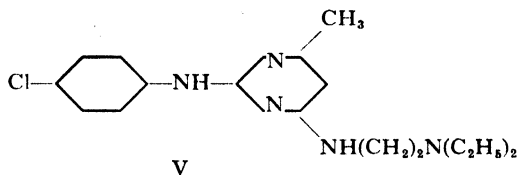
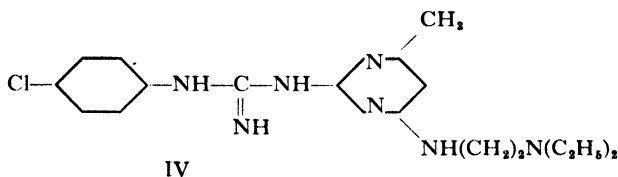
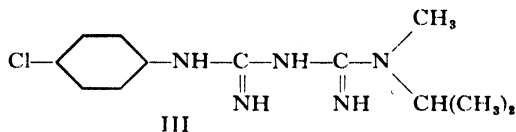
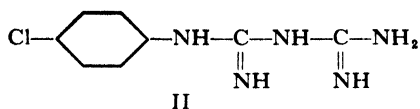
Concentrations of Paludrine in tissue are higher than those in plasma but differ according to the tissue, falling usually into the series kidney, lung, spleen, liver, intestine, pancreas, heart, muscle, fat, brain. From the first seven 10 per cent. dispersions are prepared and 5 ml. is analysed as plasma. The last three should be made into 20 per cent. dispersions and 10 ml. analysed as plasma. In the case of fat, the accuracy of analysis is lessened by an increase in volume of the benzene layer caused by solution of fat. Rough allowance for this can be made by assuming an increase in volume of the benzene-layer equal to that of the fat taken. Brain gives considerable difficulty, as lipid precipitation occurs during the final extraction with hydrochloric acid. We have found it best to centrifuge at high speed for 10 minutes, when the solid forms a cake at the solvent-acid interface. The lower layer is then withdrawn without breaking up this cake. After autoclaving, the acid is usually clear; but if some turbidity occurs it is best to extract the dye into 1–2 ml. of butanol (King, 1945) and to read against standards similarly extracted with butanol. Concentrations of Paludrine in brain are very low, and normal brain should be analysed along with the experimental sample. A blank of 0.0–0.2 mgm./kgm. is usual and should be subtracted from the experimental result. Blanks from other tissues may safely be neglected. Tissues should be stored in the refrigerator until analysis. Immersion in alcohol or formalin removes Paludrine.

EXPERIMENTAL SECTION

I. HYDROLYSIS OF PALUDRINE TO *p*-CHLOROANILINE

The hydrolysis of Paludrine (I) to *p*-chloroaniline has been examined in comparison with that of the closely related compounds 3327 (II), 4430 (III), 3349 (IV) and 2666 (V).





Since diazotized *p*-chloroaniline does not couple readily below *pH* 6, acid of greater strength than *N*/4 must be partially neutralized with sodium hydroxide in the final stage of the determination. For reasons of convenience, therefore, the hydrolysis of these compounds was examined under four sets of conditions only: in *N*/4 hydrochloric acid at 100° C. and at 20–25 lb. pressure; and in *N*/1 hydrochloric acid at 100° C. and at 20–25 lb. pressure. In the pressure hydrolyses, solutions of the five compounds of strengths 10 μ gm./ml., in the desired acid, were sealed in ampoules and autoclaved for varying periods of time. The amount of *p*-chloroaniline in a 1 ml. aliquot was determined by comparison of the developed colour with series of *p*-chloroaniline standards, prepared in the appropriate acid and simultaneously developed. In the hydrolyses at 100°, 10 ml. volumes of the drug-solutions in test-tubes, stoppered with cotton-wool plugs, were heated in a boiling-water bath and the tubes were withdrawn at intervals. After making up the volumes to 10 ml. with the appropriate acid, 1 ml. aliquots were analysed in the same manner as the pressure hydrolysates.

In no experiment was any detectable amount of *p*-chloroaniline formed from 2666, which can, however, be hydrolysed in stronger acid (2.5–5 *N*) at 20–25 lb. pressure. The results obtained with the other four compounds are illustrated in diagrams 1, 2, 3 and 4. In each experiment the order of decreasing ease of hydrolysis was 3327, Paludrine, 4430, 3349. The biguanides show increasing substitution in the same order. Paludrine was more or less completely hydrolysed in *N*/4 hydrochloric acid at 20–25 lb. pressure after three to four hours, and the latter time was therefore chosen as standard for the method. No advantage is to be gained by carrying out hydrolyses in *N*/1 hydrochloric acid, but it is clear that Paludrine can be sufficiently hydrolysed in *N*/4 hydrochloric acid at 100° C. to enable it to be determined without the use of an autoclave, if the latter is not available.

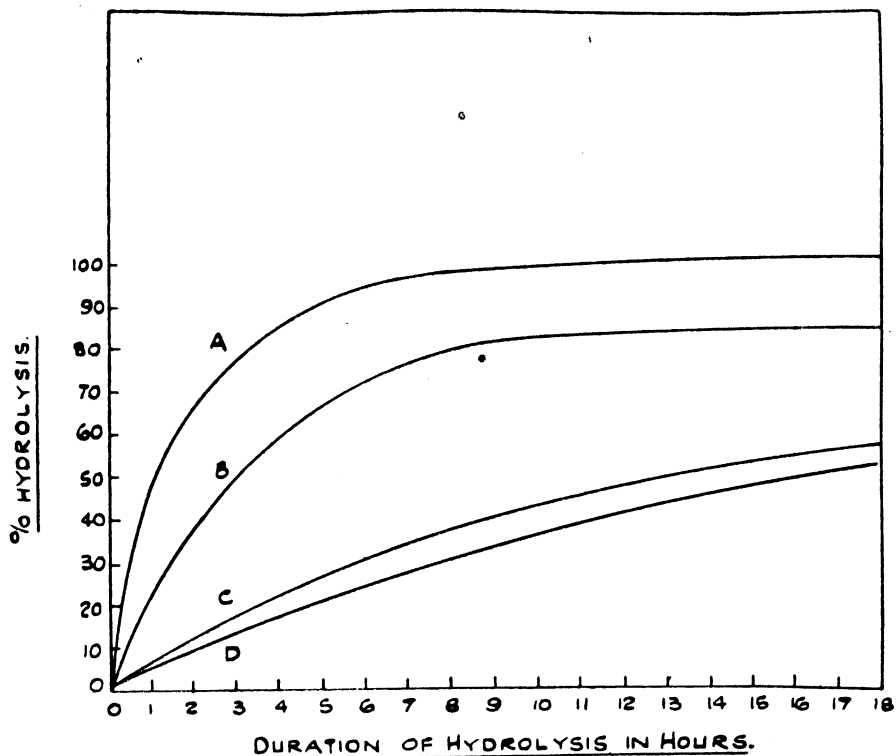


DIAGRAM 1. Hydrolysis in N/4 hydrochloric acid, at 100° C., of 3327 (A), Paludrine (B), 4430 (C) and 3349 (D).

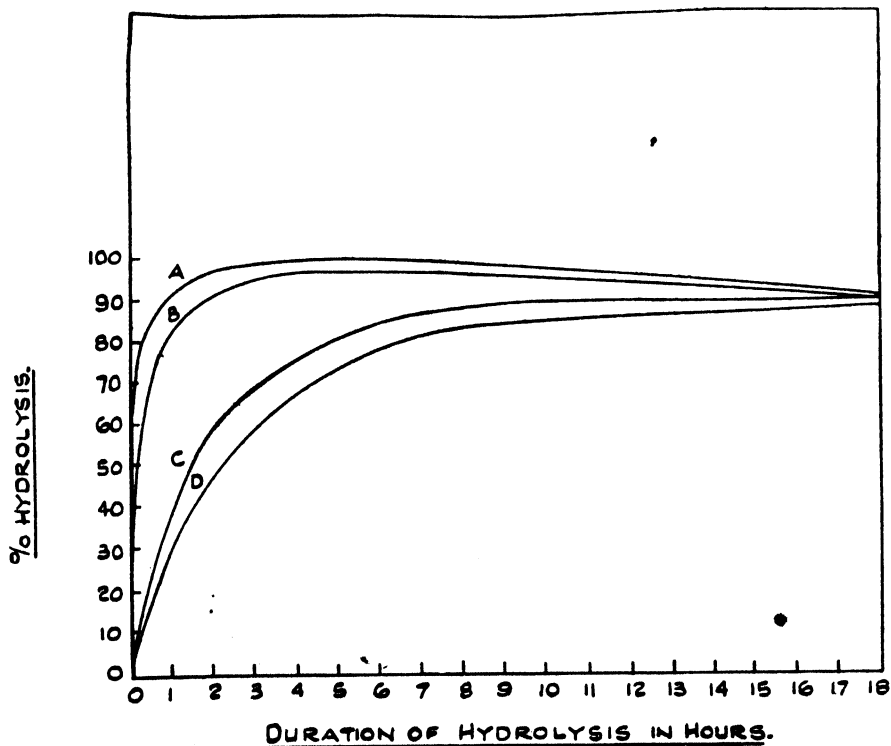


DIAGRAM 2. Hydrolysis in N/4 hydrochloric acid, at 20-25 lb. pressure, of 3327 (A), Paludrine (B), 4430 (C) and 3349 (D).

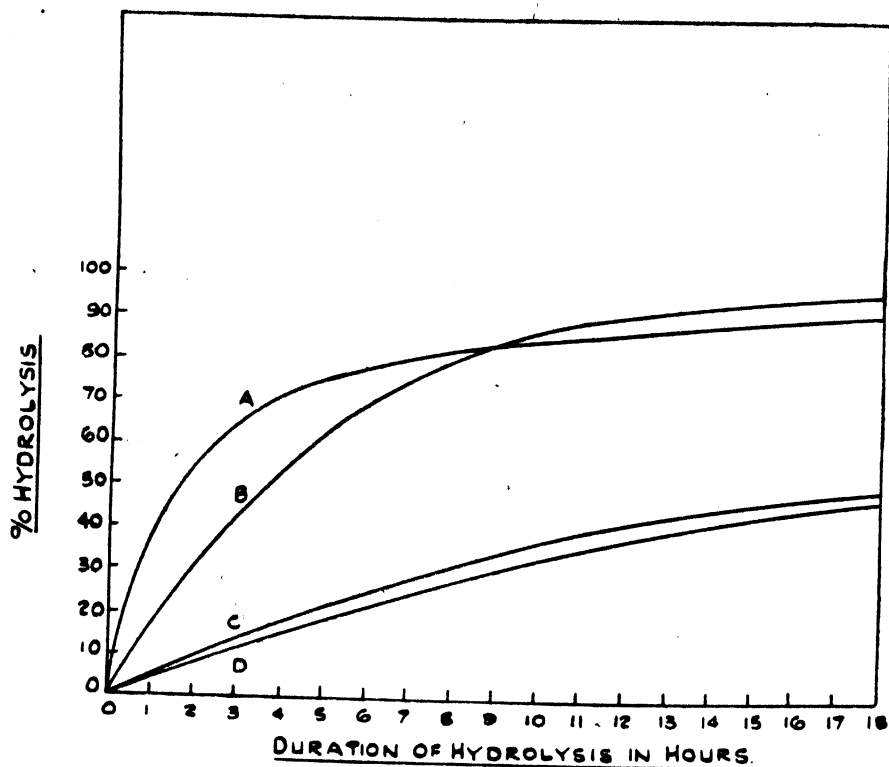


DIAGRAM 3. Hydrolysis in N/1 hydrochloric acid, at 100° C., of 3327 (A), Paludrine (B), 4430 (C) and 3349 (D).

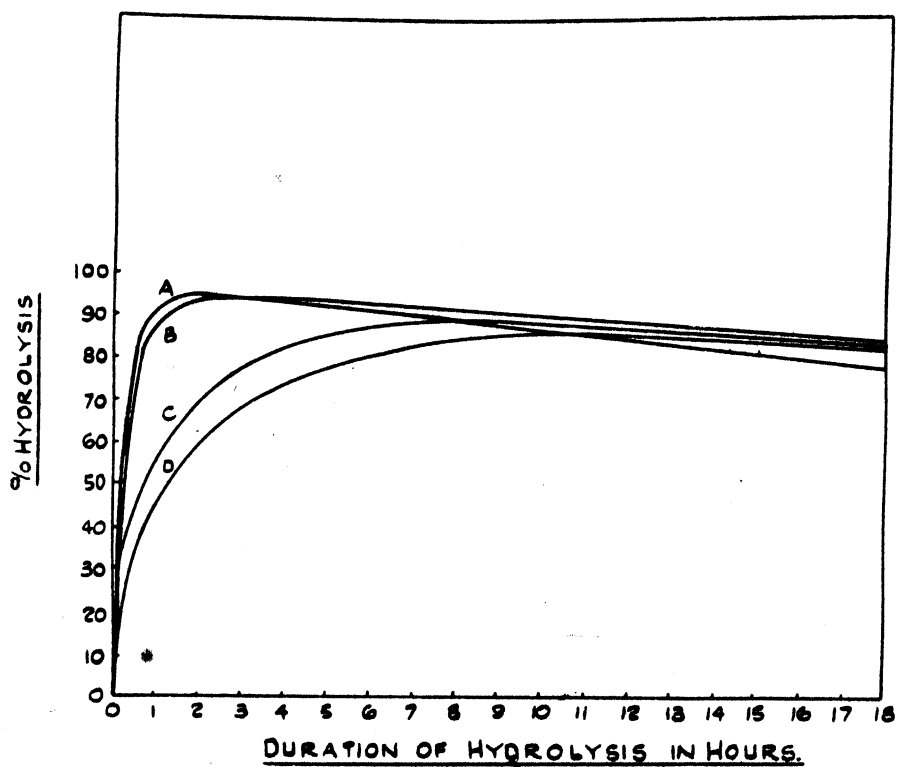


DIAGRAM 4. Hydrolysis in N/1 hydrochloric acid, at 20-25 lb. pressure, of 3327 (A), Paludrine (B), 4430 (C) and 3349 (D).

II. CHOICE OF COUPLING COMPONENT

The choice of coupling component (for sulphonamide determination as well as in the present work) lies between *N*- β -sulphatoethyl-*m*-toluidine (Rose and Bevan, 1944) and β -1-naphthylethylenediamine (Bratton and Marshall, 1939). Others that have been suggested are less satisfactory, either because of lower stability or because of the lower colour intensities of the dyes formed with diazonium salts. The advantages in favour of *N*- β -sulphatoethyl-*m*-toluidine are: (1) greater ease and cheapness of preparation;

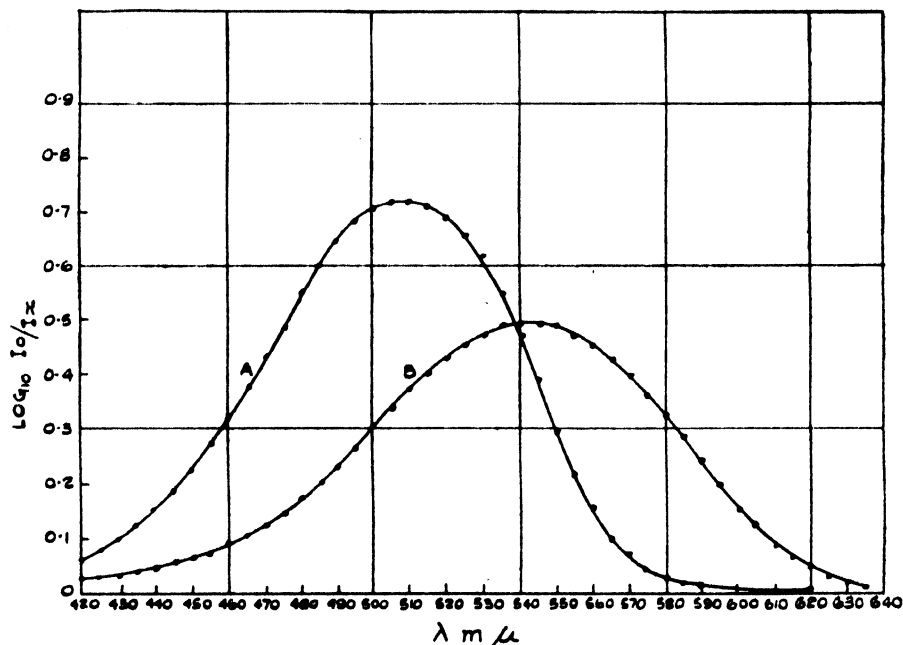


DIAGRAM 5. Absorption spectra of the azo dyes obtained on coupling diazotized *p*-chloroaniline with *N*- β -sulphatoethyl-*m*-toluidine (A) and β -1-naphthylethylenediamine (B).

(2) greater stability; (3) its use does not require an additional sulphamate addition to destroy excess nitrite; (4) the dyes formed, at least from sulphanilamide, sulphamezathine and *p*-chloroaniline, have maximum extinction coefficients ($E_{1\%}^{1\text{cm.}}$) 30-50 per cent. higher than those given by β -1-naphthylethylenediamine. The advantages in favour of β -1-naphthylethylenediamine are: (1) diazonium salts couple rather more rapidly with it than with *N*- β -sulphatoethyl-*m*-toluidine; (2) the dyes formed show absorption maxima further removed towards the red than those from *N*- β -sulphatoethyl-*m*-toluidine. The last point is important in visual colorimetry since the effect of this shift towards the red is to give a colour which, to the eye, appears bluish-red, instead of orange-red, and much more intense. That this higher intensity is only apparent is shown in diagram 5. 200 $\mu\text{gm.}$ of *p*-chloroaniline in 5 ml. of water was diazotized by addition of 1 ml. of trichloroacetic acid and 1 ml. of 0.1 per cent. sodium nitrite. After 10 minutes, 1 ml. of 0.5 per cent. sulphamic acid was added, followed after two minutes by 2 ml. either of 1 per cent. *N*- β -sulphatoethyl-*m*-toluidine or of 0.1 per cent. β -1-naphthylethylenediamine, and 1 ml. of 30 per cent. sodium acetate. After 10 minutes, 1 ml. of con-

centrated hydrochloric acid was added and the volume was made up to 100 ml. Absorption spectra of the two dye solutions were constructed, using 1 cm. cells in a Beckman quartz spectrophotometer, model DU, with a tungsten filament lamp as light-source, and exit-slits at 510 and 540 $m\mu$ of 0.065 and 0.067 mm. respectively, equivalent to nominal band widths of 1.4 and 1.7 $m\mu$. The higher maximum extinction coefficient of the dye from N- β -sulphatoethyl-*m*-toluidine points to the use of this coupling component where a photoelectric colorimeter or a visual colorimeter provided with filters (for example the Zeiss Pulfrich photometer) is available.

The method can readily be adapted for use with β -1-naphthylethylenediamine by introducing a sulphamate addition after diazotization.

III. STABILITY OF THE DYE SOLUTIONS

The stability of the dye solutions was investigated as follows: 10 μ gm. of *p*-chloroaniline in 1 ml. of N/4 hydrochloric acid was diazotized and coupled with N- β -sulphatoethyl-*m*-toluidine in the usual manner (with omission of sulphamic acid), and the volume was made up to 10 ml. The absorption spectrum was constructed using 2 cm. cells in the Beckman spectrophotometer, and the extinction coefficient at the wave-length of maximum absorption (506 $m\mu$) was determined at intervals. It remained constant for 17 hours. Diagram 6 shows the absorption spectra of the sample immediately after colour development and after 17 hours. The increased absorption at lower wave-lengths which becomes evident after such intervals is probably due to the slow formation of nitroso derivatives of N- β -sulphatoethyl-*m*-toluidine. As indicated in the description of the method, solutions should be examined within an hour of colour development unless very narrow transmission-filters or a spectrophotometer is available.

IV. IDENTITY OF PALUDRINE IN HUMAN URINE WITH THAT ADMINISTERED

It was thought important to isolate Paludrine from the urine of subjects receiving the drug in order to discover whether the amount obtained was similar to that indicated by the hydrolytic method. Two and a half litres of human urine containing 793 mgm. of Paludrine, as indicated by analysis, was evaporated *in vacuo* at 30° to 400 ml.; 20 ml. of 10N sodium hydroxide was added, and the suspension was extracted four times with 100 ml. of ether, alcohol being added as necessary to resolve emulsions. The combined ether extracts were washed twice with N/5 sodium hydroxide and once with distilled water, and were extracted three times with 20 ml. of 5 per cent. acetic acid. The combined acid extracts were basified by addition of 10 ml. of 10N sodium hydroxide and allowed to stand in the refrigerator overnight. The precipitated Paludrine was filtered, well washed with distilled water and dried *in vacuo* over calcium chloride. The residue weighed 640 mgm. and melted at 110–2° C. The melting-point of pure Paludrine, isolated from aqueous solution, varies from 90° to 130° C. according to the extent of hydration. The product was therefore crystallized from benzene to give almost pure anhydrous Paludrine (520 mgm.); melting-point 125° (uncorrected); mixed with an authentic specimen, melting-point 125° C.; found: C 51.95, H 6.1, N 27.3, Cl 14.2 per cent.; $C_{11}H_{18}N_6Cl$ requires: C 52.1, H 6.3, N 27.6, Cl 14.0 per cent. The ultra-violet absorption spectrum of the purified material in N/10 hydrochloric acid, constructed using the Beckman spectrophotometer, was almost coincident with that of an authentic specimen (diagram 7). The purity

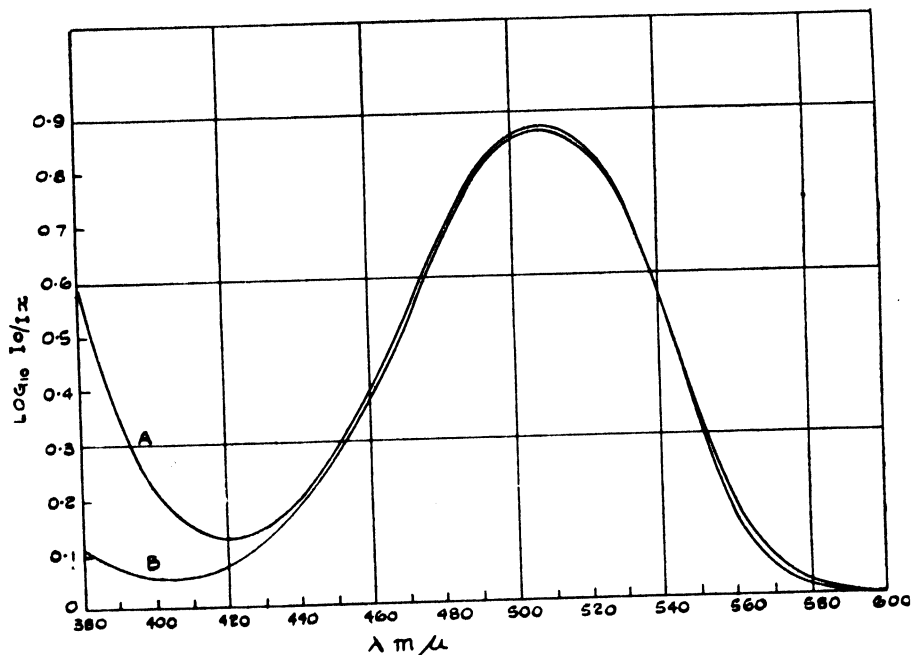


DIAGRAM 6. Absorption spectra of a solution of *p*-chloroaniline diazotized and coupled with *N*- β -sulphatoethyl-*m*-toluidine : B immediately after preparation, A after 17 hours.

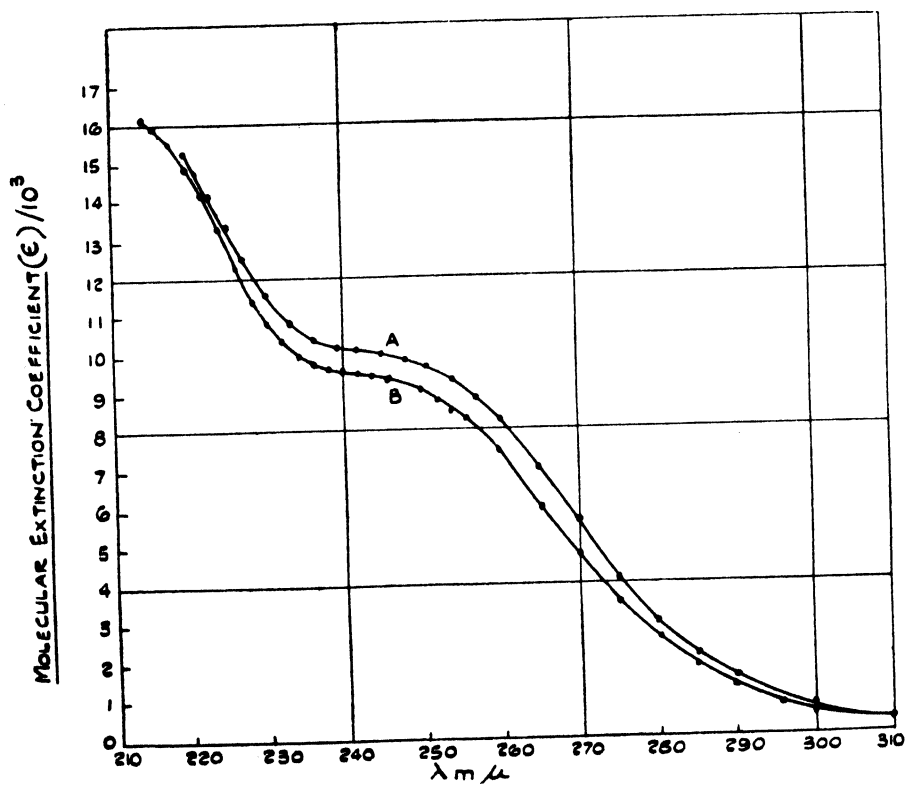


DIAGRAM 7. Absorption spectra in *N*/10 hydrochloric acid, of pure Paludrine (B) and of Paludrine isolated from human urine (A).

of the Paludrine isolated, and the fair agreement of the amount found with that expected from its determination by the hydrolytic method, indicate that the latter is probably reasonably specific.

SUMMARY

1. The hydrolytic method for the determination of Paludrine (Spinks and Tottey, 1945b) has been re-examined and modified as a result of examining the hydrolysis of Paludrine and related compounds to *p*-chloroaniline under various conditions.

2. Special precautions, suggested by about a year's experience in the use of the method, are described.

3. Reasons are given for choosing N- β -sulphatoethyl-*m*-toluidine as coupling component in the present method, and for the determination of sulphonamides.

4. Paludrine has been isolated from human urine in an amount comparable to that expected from its determination therein by the method.

ACKNOWLEDGEMENTS.—The authors wish to thank Miss R. B. Hörrocks for technical assistance.

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A TECHNIQUE FOR OBTAINING BACTERIA-FREE SUSPENSIONS OF SPOROZOITES FROM THE SALIVARY GLANDS OF INFECTED MOSQUITOES

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(Received for publication April 4th, 1946)

Huff and Coulston (1944) claim to have demonstrated the primary phase of the avian parasite *Plasmodium gallinaceum* in the domestic fowl. This observation, if confirmed, may represent an important advance in the methods at present employed in the study of the chemotherapy of malaria, since it should allow of observations being made on the 'screening' of drugs under investigation as 'true prophylactics.'

Huff's technique for the demonstration of the primary phase involves the injection of immense numbers of salivary glands, usually 200-400, from infected mosquitoes, and the subsequent examination of sections and smears made from the inoculated tissue. The value of any method which allows of a study of the primary phase of the malaria parasite is obviously great, but the very large number of mosquitoes required for Huff and Coulston's technique would appear to limit its value as a practical method of observing the development of sporozoites under various conditions.

Another, and simpler, method of studying the primary phase by the addition of the sporozoites to tissue culture has been suggested. This has been tried by ourselves and other workers, but all attempts to observe the subsequent growth of sporozoites in such tissue-cell cultures have failed. One of the reasons suggested for this failure has been that, so far, it has not been possible to obtain sporozoites free from bacteria. This difficulty has in part been overcome by the addition of penicillin to the sporozoite emulsion before adding the parasites to the tissue being cultured. In this connection we have confirmed the statement by Hawking (1945) that penicillin has no direct lethal action on sporozoites, at any rate over short periods. Unfortunately, however, penicillin also lacks any inhibitory action on fungi, while we have found that some of the bacteria present after the ordinary dissection of mosquitoes are not penicillin-sensitive. In addition, although Hawking has shown that extraerythrocytic forms of *P. gallinaceum* grow readily in tissue culture containing penicillin, similar success did not attend attempts to grow the endoerythrocytic forms in the same medium. It does not, of course, follow that the penicillin was in any way responsible for this failure, but it is obviously advantageous to avoid the introduction of any factor the harmlessness of which has not been fully proved.

Dr. D. S. Bertram and one of the writers in 1939 (reported by Bishop and Gilchrist, 1946) succeeded in rearing bacteria-free adult *Aedes aegypti*, using the method described

by Barber (1927). The resultant mosquitoes were then allowed to gorge through sterile membranes on avian blood containing *P. gallinaceum*. In this way the present authors obtained sporozoites free from bacteria or fungi; but, although successful, the method was too laborious for practical purposes, and subsequently various methods of washing the mosquitoes and then dissecting them with sterile precautions were tried.

In our early experiments we dissected the salivary glands from uninfected as well as from infected mosquitoes, and in the latter instance we did not usually test the viability of the treated sporozoites, as judged by their ability to cause infection when subsequently injected into a bird. Out of a total of 60 mosquitoes, dissected after washing with alcohol, the glands of 55 showed no growth when inoculated into Brewer's medium, while the haemocoel fluids of 28 of these were similarly sterile. It may be noted that in all instances where the haemocoel fluid was sterile the glands were also sterile.

After various modifications of our earlier methods, the following technique was devised in order to obtain healthy sporozoites free from bacteria and fungi.

A batch of *Aedes aegypti* are fed, 18 days prior to the experiment, on a fowl infected with *P. gallinaceum* and showing numerous gametocytes in the peripheral blood.

One lightly anaesthetized mosquito is held by the proboscis in forceps under a stream of rapidly falling drops of 75 per cent. alcohol for $1\frac{1}{2}$ minutes. After this preliminary washing in alcohol, the mosquito is rapidly drained on two successive slips of sterile filter-paper and transferred to a drop of sterile Tyrode's solution on a sterile slide placed under a dissecting microscope. The mosquito is then dissected with sterile needles and the salivary glands are removed without cutting the oesophagus. The dissected glands are next transferred to a fresh drop of sterile Tyrode's solution and finely minced. A rigid aseptic technique, such as that employed in tissue culture, is observed throughout all these manipulations and the subsequent inoculation of media and tissue.

If sporozoites are found to be present and motile, one platinum loopful of the emulsion is sown in 10 c.cm. of Brewer's medium.* A sample of the sporozoite emulsion is then injected into a fowl, in order to confirm that the sporozoites have not been injured by the sterilizing alcohol treatment of the mosquito.

In order to prove the presence or absence of viable bacteria in the relatively large quantity of haemocoel fluid, approximately 1 minim of the dissection fluid is sown in another tube of Brewer's medium.

All cultures are kept at 37° C. and examined daily for 10 days after inoculation.

The results shown in the table are those obtained in our last series of experiments and suggest that a high proportion (in this case 100 per cent.) of the glands obtained by this technique are free of viable bacteria and fungi, and that the treatment has no injurious effect on the sporozoites which they contain.

*The sodium thioglycollate medium recommended by Brewer (1940), and commonly used by the 'Blood Bank' for testing the sterility of plasma, consists of:

Pork infusion solids	1	per cent.
Peptone (thio)	1	" "
Sodium chloride	0.5	" "
Sodium thioglycollate	0.1	" "
Agar	0.05	" "
Glucose...	0.1	" "
Methylene blue	0.002	" "

TABLE

Date	Mosquito no.	Sporozoite concentration in salivary glands	Motility of sporozoites after sterilizing treatment of mosquito	Result of cultures; final reading on 10th day			Infectivity of sporozoites to birds after 'sterilizing' treatment of mosquito	
				Haemocoel fluid of mosquito, after removal of glands		Glands after removal from mosquito		
				Brewer's medium	Nutrient broth	Brewer's medium	Result of inoculation	Date positive
8.3.46	1	++	+	y	y	0	Positive	18.3.46
8.3.46	2	++	+	y	y	0	"	18.3.46
8.3.46	3	++	+	y	y	0	"	19.3.46
8.3.46	4	+	+	y	y	0	"	19.3.46
8.3.46	5	+	+	0	y	0	"	18.3.46
8.3.46	6	++	+	0	0	0	"	15.3.46
9.3.46	1	++	+	y	+*	0	"	20.3.46
9.3.46	2	++	+	y	y	0	"	19.3.46
9.3.46	3	++	+	0	0	0	"	19.3.46
9.3.46	4	+†	+	y	0	0	"	21.3.46
9.3.46	5	++	+	0	0	0	"	19.3.46
10.3.46	1	++	+	y	y	0	"	21.3.46
10.3.46	2	+†	+	y	y	0	"	25.3.46
10.3.46	3	+†	+	y	y	0	"	20.3.46
10.3.46	4	+++	+	0	0	0	"	20.3.46

0 = No growth. + = Growth of bacteria. y = Growth of yeast, but no growth of bacteria.
 * = Yeast also present. † = Very light infection.

ACKNOWLEDGEMENTS.—We are indebted to Professor D. T. Robinson and Dr. J. R. Holt for checking the results of our reading of the cultures.

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J. W. W. Stephens

IN MEMORIAM

The editors regret to announce the death, on May 17th, 1946, of Emeritus Professor JOHN WILLIAM WATSON STEPHENS, M.D., F.R.S.

Stephens was appointed Walter Myers Lecturer at the Liverpool School of Tropical Medicine in 1903 and succeeded Sir Ronald Ross as Alfred Jones Professor of Tropical Medicine in 1913.

His name will always be associated with certain outstanding advances in our knowledge of tropical medicine, particularly those due to his pioneer and long-continued work on malaria and blackwater fever.

He was an editor of these *Annals* from their inception in 1907 until his retirement in 1928. The standard of scientific publication which he set for himself was of so high an order that nothing which he has written has been lightly set aside, while his great knowledge of the literature and his fine critical ability proved an inspiration to all who sought his advice.

During the fifteen years that he was the senior member of the staff of the Liverpool School he served it faithfully, guided it through many difficulties and added much to its reputation.

TAXONOMY OF THE ETHIOPIAN SANDFLIES (*PHLEBOTOMUS*)

II.—KEYS FOR THE IDENTIFICATION OF THE ETHIOPIAN SPECIES

BY

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(*Received for publication November 13th, 1945*)

NOTES ON THE USE OF THE KEYS

We have omitted from this paper any account of the anatomy of *Phlebotomus* or of the nomenclature of the characters used in classification, since these matters have already been fully dealt with in papers by Newstead (1911), Sinton (1932, 1933) and Parrot (1934), which we presume will be available to anyone likely to use the keys here presented.

For the same reason we have not described methods of preparing specimens for examination. A detailed account of this technique will be found in Sinton's (1932) paper, but most workers find it advantageous to devise modifications in technique to suit the particular climatic conditions with which they are concerned. It is extremely important to obtain good clear preparations; otherwise details of structure are obscured, and identification may be impossible.

The keys given below should not be regarded as a substitute for the systematic descriptions of the various species, with which specimens should finally be compared before determination is made; better still is comparison with type-specimens where this is possible. The principal function of the keys is to facilitate the exclusion of irrelevant matter, which is otherwise difficult at present owing to the scattered and extensive nature of the literature. With this object in mind we have omitted varieties from the keys. The main points of difference between each variety and the type-species have been noted in the previous section, which can be consulted, where necessary, after the species has been determined.

Some points from the preceding section of this paper (which we hope will be a useful adjunct to the keys) may be summarized briefly.

1. The following species, which are excluded from the keys, are of doubtful status in the present state of knowledge:

P. antennatus Newstead

P. bedfordi Newstead

P. duboscqi Neveu-Lemaire

2. The following Palaearctic species, which are also excluded from the keys, have been erroneously recorded from the Ethiopian region:

P. perniciosus

P. tiberiadis

P. minutus

3. The following are *nomina nuda*, having become synonyms as shown below :

<i>P. brodeni</i>	= <i>P. simillimus</i>
<i>P. ghesquieri</i>	= <i>P. squamipleuris</i>
<i>P. mathisi</i>	= <i>P. buxtoni</i>
<i>P. nairobiensis</i>	= <i>P. congolensis</i>
<i>P. sanneri</i>	= <i>P. signatipennis</i>
<i>P. symesi</i>	= <i>P. schwetzi</i>
<i>P. vagus</i>	= <i>P. clydei</i>
<i>P. viduus</i>	= <i>P. papatasii</i> var. <i>bergeroti</i>

4. The females of *P. rodhaini*, *P. rossi*, *P. katangensis*, *P. viator* and *P. meilloni* var. *suberectus* are still unknown.

The males of *P. transvaalensis*, *P. caffaricus*, *P. notatus*, *P. africanus* vars. *ater*, *magnus* and *meridianus*, *P. freetownensis*, *P. renauxi*, *P. yvonnae*, and *P. squamipleuris* vars. *dreyfussi* and *inermis* are still unknown.

Parrot and Jolinière (1945) have recently reported two new species, *P. hirtus* (♂) and *P. eremitis* (♂♀), from Hoggar, which, although near the northern border, is usually regarded as outside the Ethiopian region. Most of the species of *Phlebotomus* occurring in Hoggar are Palaearctic ones, but some Ethiopian species, like *P. schwetzi* and *P. papatasii* var. *bergeroti*, have been found there. We have not included *P. hirtus* and *P. eremitis* in this paper, but it is possible that they may be shown later to be Ethiopian rather than Palaearctic species. *P. eremitis* closely resembles *P. africanus*, while *P. hirtus* ♂ is distinguished by having seven spines on the distal segment of the superior clasper.

For convenience in identification we have often found it useful to regard several closely allied forms as constituting a sort of ill-defined group within the subgenus. Thus in the subgenus *Phlebotomus* it is possible to recognize the *papatasii* group, the *sergenti* group and the *major* group, which are distinguished by the characters of the male genitalia. Three of the Ethiopian sandflies, *P. katangensis*, *P. martini* and *P. rossi*, are rather intermediate between the *major* and *sergenti* groups. Similarly, in the subgenus *Prophlebotomus* we have found it useful to group the species as follows :

1. *Signatipennis* group, including *P. signatipennis*, *P. occidentalis*, *P. antennatus*, *P. congolensis* and its varieties, *P. buxtoni*, *P. schoutedeni* and *P. yusafi*, all having simple tubular spermathecae (fig. 9) and a heavily armed pharynx in the female (figs. 30, 31), and a blunt curved intromittent organ in the males (fig. 18).

2. *Africanus* group, with elliptical capsular spermathecae (fig. 8), including *P. africanus* and its varieties, *P. freetownensis*, *P. yvonnae*, *P. simillimus* and possibly *P. bedfordi*. Of these species, *P. simillimus* has a heavily armed pharynx of the *signatipennis* type (fig. 29), but this feature is not seen in the other members of the group.

3. *P. collarti* and *P. decipiens* may be grouped together on the character of the spermathecae (fig. 7).

4. Among the remaining species *P. squamipleuris* and its varieties, *P. wurtzi* and *P. mirabilis* constitute a rather anomalous group, having features not found in other members of the subgenus *Prophlebotomus*. *P. schwetzi* and its varieties have the simple tubular spermatheca and characteristic intromittent organ of the *signatipennis* group, but the pharynx is distinctive (fig. 28).

We do not consider that these groups have any taxonomic status, but they are often very useful to remember when one is engaged in the determination of specimens.

Finally, we would emphasize again that the following keys should be used in conjunction with the systematic descriptions of the various species, to which full references will be found in the preceding section of this paper.

THE KEYS

KEY FOR THE FEMALES OF THE SUBGENUS *PHLEBOTOMUS*

1. Females with numerous erect hairs on dorsal aspect of abdominal segments II-VI, usually in tufts at the distal end of the segment. Spermathecae segmented, except in *P. gigas*. Buccal armature and pigmented area absent or rudimentary ... 2
2. Very large species. Alar index (α/β) = 5 or 6. Spermatheca not segmented, but open into vulva by a wide common duct. Peculiar arrangement of antennal geniculate spines. A specialized cave-dwelling species ... *P. gigas*
Spermatheca segmented in entire length ... 3
3. Spermatheca with a long neck (fig. 2). Pharyngeal armature consists of rows of very small point-like teeth (fig. 25) ... 4
Spermatheca not provided with long neck ... 5
4. Spermatheca with 10-12 segments. The terminal (non-annulated) dilated part of spermathecal duct long ($=\frac{1}{2}$ of total length of duct; fig. 2) ... *P. langeroni* var. *orientalis*
Spermatheca with 12-14 segments. Terminal (non-annulated) dilatation of spermathecal duct short ... *P. longipes*
5. Pharyngeal armature composed of coarse teeth (fig. 26). Spermatheca with only 4-6 segments, without expanded head ... *P. sergenti*
P. alexandri
Spermatheca with more than 5 segments. Pharyngeal teeth less marked ... 6
6. Pharyngeal armature composed of fine ridges and small punctiform teeth. Spermatheca has 9-10 segments (fig. 3) ... *P. martini*
Pharyngeal armature appearing as a network of fine wavy lines. Spermatheca with 8-12 segments, and large expanded head, with no neck (fig. 4) ... *P. papatasi*
P. roubaudi
P. duboscqi

KEY FOR THE MALES OF THE SUBGENUS *PHLEBOTOMUS*

1. Males with numerous erect hairs on abdominal tergites II-VI. Buccal armature and pigmented area absent or rudimentary. Genitalia usually having a specific morphology ... 2
2. Proximal segment of superior clasper bearing on its inner aspect a prominent tuft of hairs arising from a peduncle (figs. 10, 11) ... 3
No such tuft of hairs ... 7
3. Distal segment of superior clasper bearing 4 spines (fig. 11) ... 4
Distal segment of superior clasper bearing 5 spines (fig. 10) ... 5
4. One of the two distal spines on the distal segment of superior clasper is apical, the other subapical (as in fig. 11). Peduncle bearing the tuft of hairs on proximal segment is short ... *P. alexandri*
The two distal spines on distal segment of superior clasper are both terminal (except in var. *saevus*, in which the peduncle bearing the tuft of hairs is elongated; fig. 11) ... *P. sergenti*

5. Peduncle on proximal segment of superior clasper bearing long hairs of uniform length and shape *P. katangensis*
Peduncle bearing hairs of different length and shape 6
6. Peduncle on proximal segment of superior clasper bearing about 4 very long bristles apically and a large number of shorter hooked bristles ventrally, the latter decreasing in size from near the apex to the base *P. rossi*
Peduncle bears a tuft of 6 large flat hairs and about 12 shorter slender hairs, with no regular gradation in size *P. martini*
7. Distal segment of superior clasper bearing 4 spines 8
Distal segment of superior clasper bearing 5 spines 9
8. Intermediate appendage bilobed, with characteristic morphology (fig. 13), one lobe being membranous and covered with hairs, the other strongly chitinated and pigmented at the tip *P. rodhaini*
Intermediate appendage single, finger-shaped *P. gigas*
9. Distal segment of superior clasper very elongated, having parallel sides and bearing 5 short spines. Inferior clasper bearing 2-6 apical spines. Intermediate appendage with 3 characteristic lobes (fig. 14) 10
Distal segment of superior clasper irregular in shape, with 5 long spines, 2 apical and 3 submedian (fig. 12). Inferior clasper unarmed; intermediate appendage simple 11
10. Inferior clasper with 4-6 very short terminal spines (fig. 16) *P. roubaudi*
Inferior clasper with 2 long terminal spines (fig. 14) *P. papatasi*
11. Genitalia of *major* type (fig. 12); intermediate appendage with expanded distal end cut away ventrally; single point of intromittent organ directed dorsally *P. longipes*
Genitalia of *major* type; intermediate appendage less expanded and more rounded at distal end; single point of intromittent organ directed infero-externally (fig. 12) *P. langeroni* var. *orientalis*

KEY FOR THE FEMALES OF THE SUBGENUS *SINTONIUS*

1. Females having both erect and recumbent hairs on dorsal aspects of abdominal segments II-VI, well-developed buccal armature, and crenulated spermathecae, with short neck (fig. 5) 2
2. Superior claspers (cerci) long and narrow. Sternite of 8th abdominal segment with a number of stout, very long bristles arising from its posterior border (fig. 56) *P. transvaalensis*
These features not present 3
3. Buccal armature consisting of a single row of teeth; no anterior punctiform teeth 7
Anterior teeth present (fig. 40) 4
4. Several (three or more) rows of anterior teeth (fig. 40) *P. adleri*
One, or at most two, rows of anterior teeth 5
5. Buccal armature consists of about 15 widely spaced, large, sharply pointed and spike-like teeth. One or two rows of anterior punctiform teeth (fig. 41). Pharyngeal armature consists of numerous small spines carried on transverse ridges, restricted to the posterior part of the pharynx *P. clydei*
Buccal teeth not widely spaced, but either broad and contiguous, or narrow, straight and parallel, in palisade formation 6

6. Buccal teeth about 18, broad and contiguous, the lateral ones broader than the median ones and pointing obliquely towards the mid line. Two rows of anterior punctiform teeth. Pharynx unarmed ... *P. subtilis*
 Buccal cavity armed with a row of about 40 narrow, straight, pointed teeth. One row of anterior punctiform teeth, difficult to see owing to the very dark pigmented area. Pharynx very wide posteriorly ($4\frac{1}{2}$ times anterior width), armed with numerous fine teeth posteriorly and two irregular rows of strong, short spines along the posterior margin of the dorsal plate. Except for the posterior part bearing the fine teeth the whole organ is heavily pigmented ... *P. caffaricus*
7. Buccal teeth less than 20 in number, sharply pointed, with points widely spaced. Resembles *P. clydei* (fig. 41), but without anterior teeth ... *P. meillonii*
 Buccal teeth 40 or more, straight, parallel, arranged in a palisade. Pigmented area extending across whole width of buccal cavity (as in fig. 42) ... 8
8. Spermatheca with 8 segments. Buccal teeth, 40. Pharyngeal armature well developed, consisting of long, thin, backwardly directed spines (fig. 27) ... *P. affinis*
 Spermatheca with 10-12 segments. Buccal teeth 50-60. Pharyngeal armature inconspicuous, consisting of minute spines restricted to the posterior part ... 9
9. Buccal teeth 55-60. Pigmented area in the form of a segment of a circle, convex anteriorly, with a pointed anterior triangular process *P. thomsoni*
 Buccal teeth about 50. Pigmented area consists of a broad triangular part, with a blunt irregular anterior process and with a number of corrugated lines converging into the narrow anterior part ... 10
10. Spines present on anterior and posterior femora ... *P. wansoni*
 No spines on femora ... *P. matadiensis*

KEY FOR THE MALES OF THE SUBGENUS *SINTONIUS*

1. Males having both erect and recumbent hairs on abdominal tergites II-VI; buccal armature well developed and with specific morphology; genitalia of the *minutus* type, usually with hooked intermediate appendage and pointed intromittent organ (fig. 17) ... 2
2. Palpal formula 1, 2, 4, 3, 5. Anterior punctiform teeth present in buccal armature ... 3
 Palpal formula 1, 2, 3, 4, 5. Buccal armature without anterior punctiform teeth (except in *P. wansoni*) ... 5
3. Only one row of anterior punctiform teeth in buccal cavity ... 4
 Two or three rows of small anterior punctiform teeth. The main (posterior) row of buccal teeth has 10-12 teeth in a straight line (fig. 34). Pharyngeal armature inconspicuous, consisting of only some faint ridges in the posterior part of the organ ... *P. adleri*
4. Buccal armature a row of small teeth, separated into small groups of 3-6 teeth, the central ones being larger than the lateral ones. Anterior to this is an irregular row of about 6 small punctiform teeth (fig. 35) ... *P. clydei*
 Buccal armature consisting of 13-14 teeth, the lateral ones being longer than the central ones, and having their points directed towards the mid line. Anterior teeth in two lateral groups of 6-8 teeth; between these a group of small punctiform teeth ... *P. subtilis*

5. Buccal armature consisting of 18-22 equal teeth, with an anterior row of 10-12 small punctiform teeth at the bases of the teeth of the main row. Pigmented area central and compact, roughly triangular. Anterior and posterior femora bearing 8 short spines on the inner surface of the proximal part ... *P. wansoni*
No anterior teeth present in buccal cavity. Palp 1, 2, 3, 4, 5 ... 6
6. Four spines on the distal segment of the superior clasper all terminal 7
Two of the four spines on the distal segment of the superior clasper markedly subterminal, the other two being terminal. Small non-deciduous seta arises distally near origin of terminal spines ... 8
7. Non-deciduous seta on distal segment of superior clasper situated approximately at middle of segment. Buccal armature of about 16 blunt teeth, the inner teeth being parallel to each other, the outer ones inclining towards the mid line (fig. 36). Alar index <unity ... *P. affinis*
Non-deciduous seta on distal segment of superior clasper situated on the distal third of the segment. Alar index >unity. About 25 narrow parallel teeth in buccal cavity. Pigmented area rounded, central, as in *P. affinis*, but with a small knob-like anterior process *P. matadiensis*
8. Pigmented area broad, triangular, with apex directed anteriorly and base extending across whole width of buccal cavity. Buccal armature has about 35 parallel teeth ... *P. thomsoni*
Pigmented area small, central, turnip-shaped. Spines on distal segment of superior clasper are spatulate, and the non-deciduous seta arises near the level of the subterminal spines. Buccal armature has 10 broad teeth with serrated edges ... *P. meillon*

KEY FOR THE FEMALES OF THE SUBGENUS *PROPHLEBOTOMUS*

1. Species with no erect hairs (except in *P. squamipleuris*) on abdominal tergites II-VI. Buccal armature well developed and with specific morphology. Spermathecae not crenulated ... 2
2. Well-marked notch in posterior border of buccal plate (fig. 55) just anterior to buccal teeth, which number about 30 ... *P. babu*
No such notch present ... 3
3. With simple tubular spermathecae (fig. 9) ... 11
With spermathecae in the form of capsules or rounded sacs ... 4
4. Spermathecae elongated, cylindrical or elliptical capsules with smooth outline (figs. 7 and 8) ... 6
Spermathecae having a special shape, sac-like or turnip-shaped ... 5
5. Spermathecae turnip-shaped, with a series of transverse rows of short spines (fig. 6). Buccal armature with convexity directed backwards and having marked lateral protuberances (fig. 43). Pigmented area long and oval or hemispherical ... *P. squamipleuris*
Spermathecae in the form of sacs, with many fine parallel transverse ridges. Buccal cavity without pigmented area, provided with 3 pointed median teeth, and on either side a number of small irregularly arranged punctiform teeth ... *P. wurtzi*
Spermathecae in the form of sacs, constricted in the middle. Buccal teeth 12-14, pointed, arranged fanwise, the median teeth being larger than the lateral ones ... *P. mirabilis*
6. Spermathecae cylindrical capsules with parallel sides (fig. 7) ... 7
Spermathecae oval or elliptical capsules (fig. 8) ... 8

7. 10-12 equal and pointed teeth in buccal cavity, with a row of anterior punctiform teeth, each of which is placed at the interval between two teeth. Pharynx three times as wide posteriorly as anteriorly ... *P. collarti*
 12-14 unequal buccal teeth, the median ones being much narrower than the lateral ones, which are very wide (fig. 44). No anterior teeth. Pharynx slender ... *P. decipiens*
8. Pharynx heavily armed and cordiform and pigmented (fig. 29) ... *P. simillimus*
 Pharynx not cordiform and pigmented ... 9
9. 10 pointed buccal teeth. Pigmented area in the form of an elongated trapezoid, with the greater width anterior. No anterior punctiform teeth ... *P. yvonnae*
 20-65 parallel buccal teeth, arranged in a palisade, slightly concave behind. Pigmented area well developed, having a characteristic shape, broad and sausage-shaped posteriorly, with an anterior triangular prolongation ... 10
10. Ant. iii = iv + v. Alar index > unity. No anterior teeth in buccal cavity ... *P. freetownensis*
 Ant. iii < iv + v. Alar index < unity. Anterior teeth variable in buccal cavity. (Typical buccal cavity and pharynx shown in figs. 46 and 32) ... *P. africanus* and vars.
11. Pharynx cordiform (fig. 30). Fourth palpal segment longer than third ... 12
 Pharynx not cordiform ... 13
12. Buccal teeth 22-26. Pigmented area in the form of a straight band or ellipse, with an anterior helmet-shaped prolongation (fig. 47) ... *P. signatipennis*
 Buccal armature resembles *P. signatipennis*, but the teeth number 34-37 and are very straight and narrow ... *P. occidentalis*
 Buccal teeth 16-20 ... *P. cinctus*
13. Only one row of teeth in buccal cavity ... 17
 Buccal armature with more than one row of teeth ... 14
14. Posterior row of buccal armature has 30-45 teeth ... 15
 Posterior row of buccal armature has less than 15 teeth ... 16
15. Buccal cavity with 3 rows of teeth, the posterior containing about 30 teeth arranged in an arc convex in front, the median teeth being straight and narrow and forming a backwardly projecting salient in the middle of the arc (fig. 48). Two rows of anterior teeth containing 8-10 short teeth, narrow and parallel. Pigmented area faint or absent ... *P. ingrani*
 Posterior row of buccal teeth bears about 45 long pointed monomorphic teeth, arranged in an arc strongly concave posteriorly; anterior to this are at least two rows of small punctiform teeth. Pigmented area light brown, with a notch in its posterior border (fig. 49) ... *P. serratus*
16. Posterior row has 9 pointed teeth, the median teeth being shorter than the lateral ones. Anterior to this, one row of 7 strong subconical teeth ... *P. notatus*
 Posterior row with 12 pointed teeth. Anterior to this, 2 rows of small punctiform teeth having 16 and 7 teeth respectively ... *P. durenii*
17. Buccal teeth 58-60, equal and monomorphic ... *P. renauxi*
 Buccal teeth less than 40 ... 18

18. Pharyngeal armature a series of transverse parallel lines, without true teeth (fig. 28). Buccal armature distinctive (fig. 50) ... *P. schwetzi*
 Pharyngeal armature with well-developed teeth (as in figs. 31, 33) ... 19
19. Buccal teeth 20 or less ... 20
 Buccal teeth 24-40 ... 21
20. Buccal armature with 13-15 teeth, in an arc concave posteriorly.
 The four lateral teeth on each side very broad, with their points directed towards the mid line, the middle teeth being straight, narrow and parallel (as in *P. decipiens*; fig. 44). Pigmented area elliptical, with ragged posterior margin and a short anterior process ... *P. buxtoni*
 Buccal teeth 20 in number, the inequality between median and lateral teeth being much less than in *P. buxtoni*. Pigmented area dark, in the form of an ellipse without anterior prolongation (fig. 51) ... *P. schoutedeni*
21. Buccal teeth 35-40, on an arc slightly concave posteriorly, the median teeth being sometimes slightly narrower and shorter than the lateral ones. Pigmented area irregularly elliptical, with ragged posterior margin (figs. 52, 53). Pharyngeal armature very conspicuous (fig. 31) ... *P. congolensis*
 Buccal cavity with 25 teeth, on an arc strongly concave posteriorly (fig. 54). Pharyngeal armature less prominent (fig. 33) ... *P. yusafi*

KEY FOR THE MALES OF THE SUBGENUS *PROPHLEBOTOMUS*

1. Males having teeth in the buccal cavity (except in *P. wurtzi*), and no erect hairs on abdominal tergites II-VI (except sometimes in *P. squamipleuris*) ... 2
2. Distal segment of superior clasper bearing only 2 spines ... *P. mirabilis*
 Distal segment bearing 4 spines ... 3
3. No teeth or pigmented area in buccal cavity. Pharynx unarmed ... *P. wurtzi*
 Buccal armature present ... 4
4. Intromittent organ curved, finger-shaped, with blunt end (fig. 18) ... 5
 Intromittent organ conical and tapering (figs. 19, 20) ... 10
5. Two of the spines on the distal segment of superior clasper are apical, the other two arise at junction of middle and distal thirds of segment; the non-deciduous seta arises distally to the subapical spines (fig. 21). Palpal formula 1, 2, 4, 3, 5 ... *P. schwetzi*
 All four spines on distal segment of superior clasper terminal or subterminal ... 6
6. Palpal formula 1, 2, 4, 3, 5. Buccal cavity shown in fig. 38 ... *P. signatipennis*
 Palpal formula 1, 2, 3, 4, 5 ... *P. occidentalis* ... 7
7. Intermediate appendage as long, or nearly as long, as inferior clasper ... *P. yusafi*
 Inferior clasper longer than intermediate appendage ... 8
8. 24-40 teeth in buccal cavity ... *P. congolensis*
 Less than 20 teeth in buccal cavity ... 9
9. About 14 teeth in buccal cavity, the lateral ones being larger than median ones and pointing towards the centre. $A_{iii}/E < \text{unity}$... *P. buxtoni*
 16-18 teeth in buccal cavity, the difference between the median and lateral teeth not being marked. $A_{iii}/E > \text{unity}$... *P. schoutedeni*

10. Intromittent organ tapering to a sharp point (fig. 19). Buccal teeth 20-60, no pigmented area present (fig. 37). Proximal segment of superior clasper less than twice as long as distal segment, which has non-deciduous seta inserted about its mid point (fig. 24) ... *P. africanus*
 Intromittent organ tapering to a blunt point (fig. 20) ... 11
11. Intermediate appendage with hooked extremity (as in figs. 17, 24) 14
 Intermediate appendage does not terminate in a hook (figs. 22, 23) 12
12. Two of the spines on the distal segment of the superior clasper are terminal, and the other two arise from the middle of the segment. Non-deciduous seta arises proximally to the proximal spines (fig. 23) ... *P. ingrami*
 All four spines on distal segment of superior clasper terminal or slightly subterminal ... 13
13. Aiii/E > unity. Pigmented area absent ... *P. similimus*
 Aiii/E < unity. Buccal cavity with marked lateral protuberances anterior to the buccal teeth. No geniculate spines on Aiii. Genital filaments with dilated ends. Wide scales on thoracic pleurae ... *P. squamipleuris*
14. Pigmented area absent ... 15
 Pigmented area present ... 16
15. Buccal teeth 8-10. Pharynx unarmed ... *P. durenii*
 Buccal teeth 10-12, with an anterior row of small punctiform teeth. Pharynx armed with fine transverse lines ... *P. collarti*
16. Buccal teeth about 50 ... *P. viator*
 Buccal teeth less than 20 ... 17
17. Buccal teeth markedly unequal, 12-14 in number, the 4-5 median teeth being pointed and narrower than the lateral ones (as in the female of the same species; fig. 44) ... *P. decipiens*
 Buccal cavity rather resembling that of *P. signatipennis*, with 16-18 teeth, and a row of small teeth anterior to the main row ... *P. babu*

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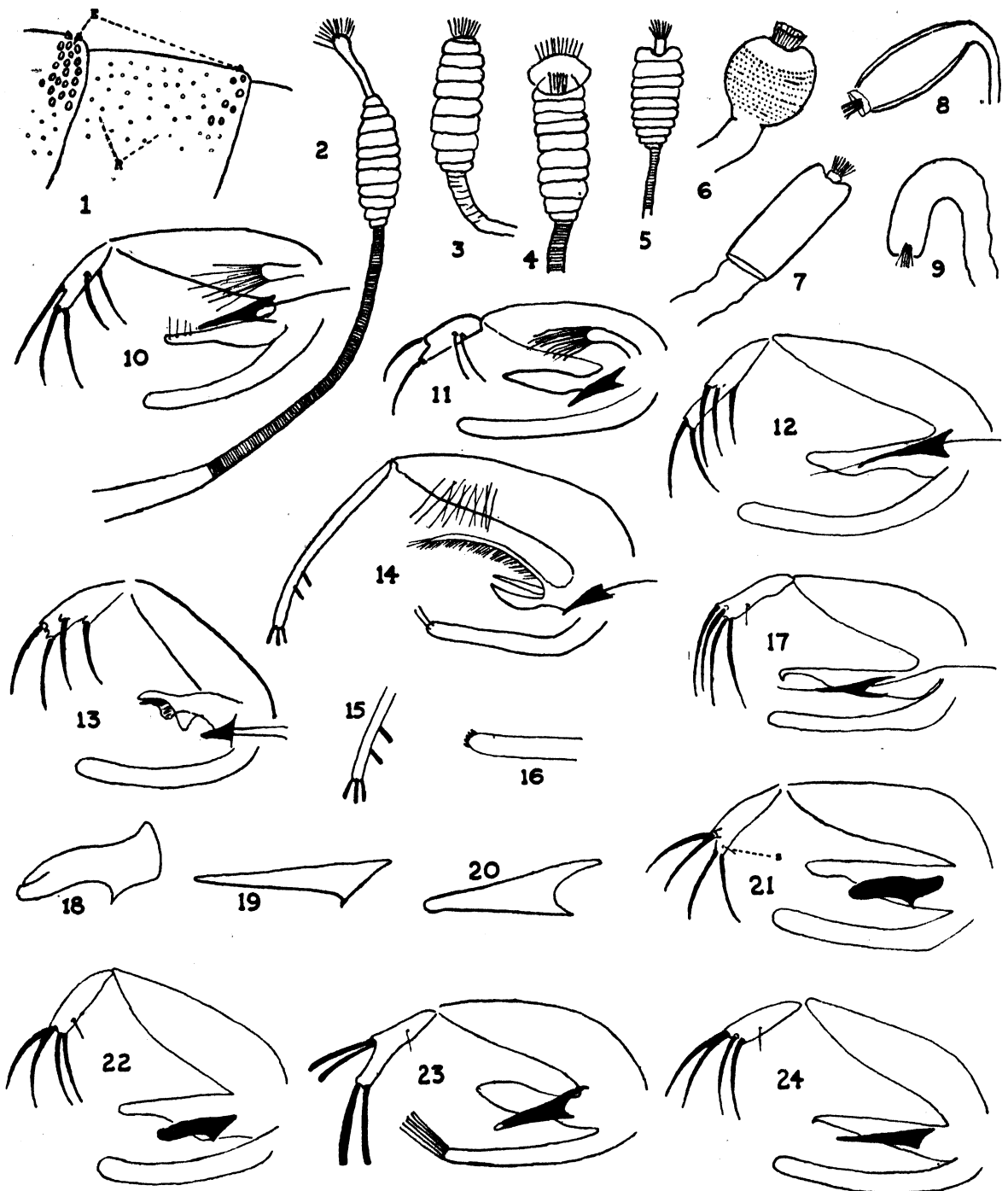


FIG. 1. Lateral view of dorsal part of first and second abdominal segments of *P. clydei*, showing scars left by erect and recumbent hairs; E = scars of erect hairs, R = scars of recumbent hairs. FIG. 2. Spermatheca of *P. langeroni* var. *orientalis*. FIG. 3. Spermatheca of *P. martini*. FIG. 4. Spermatheca of *P. papatasii*. FIG. 5. Spermatheca of *P. clydei* (typical of subgenus *Sintoniuss*). FIG. 6. Spermatheca of *P. squamipleuris*. FIG. 7. Spermatheca of *P. decipiens*. FIG. 8. Spermatheca of *P. africanus*. FIG. 9. Spermatheca of *P. congoensis*. FIG. 10. Terminalia of *P. martini*. FIG. 11. Terminalia of *P. sergenti* var. *saevus*. FIG. 12. Terminalia of *P. langeroni* var. *orientalis*. FIG. 13. Terminalia of *P. rodhaini*. FIG. 14. Terminalia of *P. papatasii*. FIG. 15. Distal segment of superior clasper of *P. papatasii* var. *bergeroti*. FIG. 16. Distal end of inferior clasper of *P. roubaudi*. FIG. 17. Terminalia of *P. clydei* (typical of subgenus *Sintoniuss*). FIG. 18. Blunt intromittent organ of *P. schwetzi*. FIG. 19. Conical intromittent organ of *P. africanus*, with sharp point. FIG. 20. Conical intromittent organ of *P. ingrani*, with blunt point. FIG. 21. Terminalia of *P. schwetzi*; s = non-deciduous seta. FIG. 22. Terminalia of *P. signatipennis*. FIG. 23. Terminalia of *P. ingrani*. FIG. 24. Terminalia of *P. africanus*.

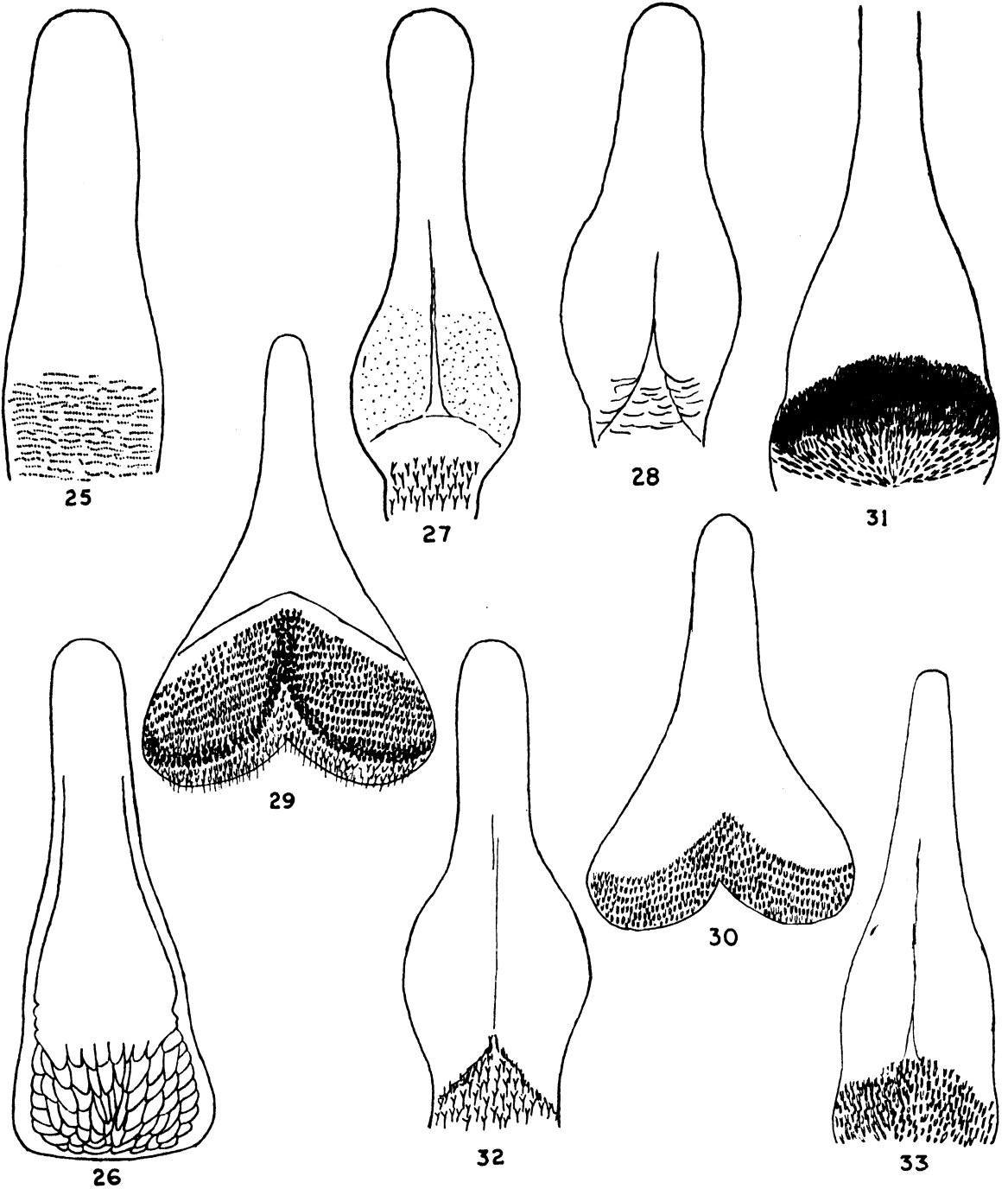
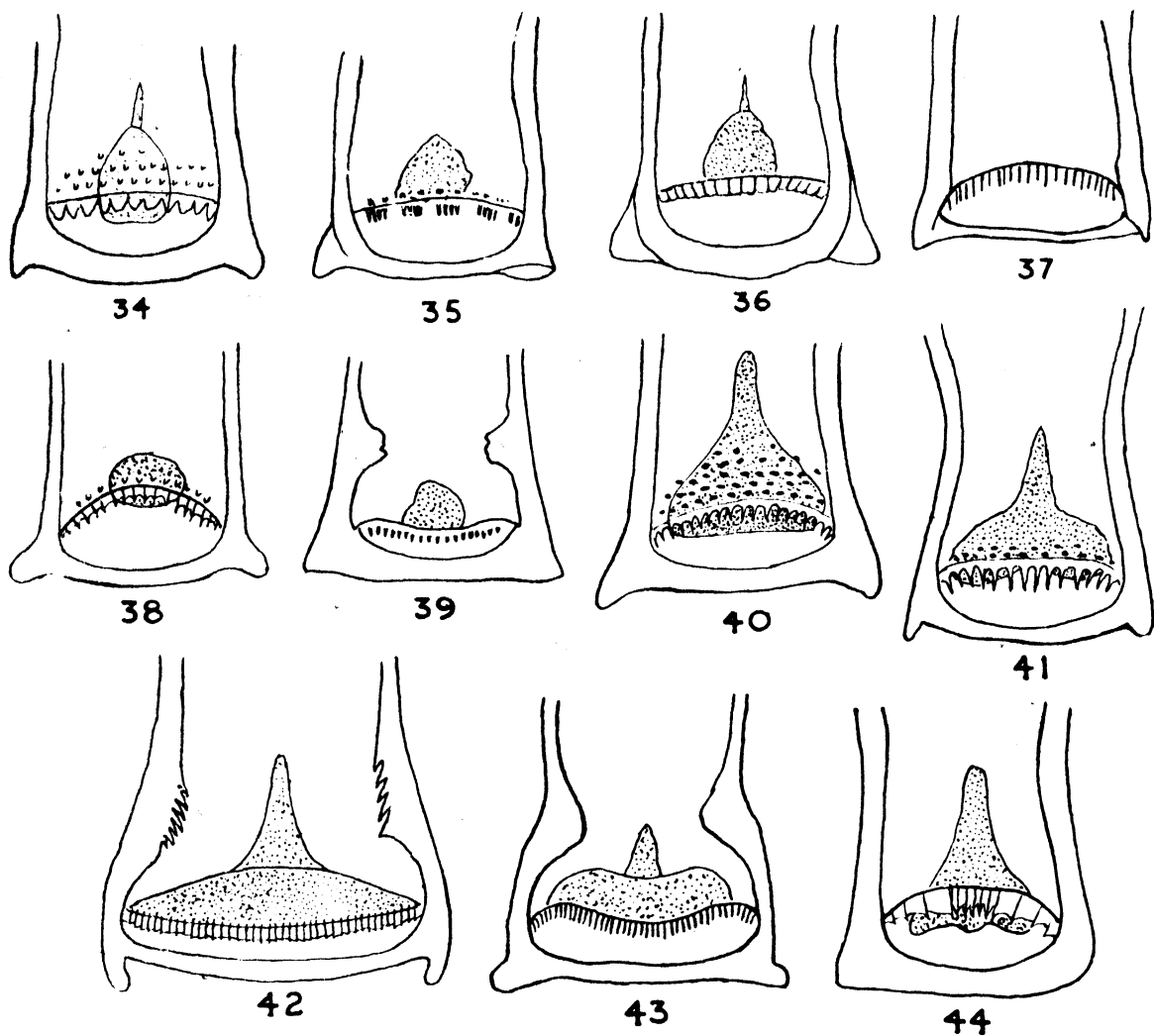


FIG. 25. Pharynx of *P. langeroni* var. *orientalis* (female).
 FIG. 26. " " *P. sergenti* (female). (Reproduced from the *Ann. Trop. Med. & Parasitol.*,
 vol. 23, p. 274.)
 FIG. 27. " " *P. affinis* (female).
 FIG. 28. " " *P. schwetzi* (female).
 FIG. 29. " " *P. simillimus* (female).
 FIG. 30. " " *P. signatipennis* (female).
 FIG. 31. " " *P. congolensis* (female).
 FIG. 32. " " *P. africanus* (female).
 FIG. 33. " " *P. yusafi* (female).



- FIG. 34. Buccal armature of *P. adleri* (male).
 FIG. 35. " " *P. clydei* (male).
 FIG. 36. " " *P. affinis* (male).
 FIG. 37. " " *P. africanus* (male).
 FIG. 38. " " *P. signatipennis* (male).
 FIG. 39. " " *P. squamipleuris* (male).
 FIG. 40. " " *P. adleri* (female).
 FIG. 41. " " *P. clydei* (female).
 FIG. 42. " " *P. affinis* (female).
 FIG. 43. " " *P. squamipleurus* (female).
 FIG. 44. " " *P. decipiens* (female).

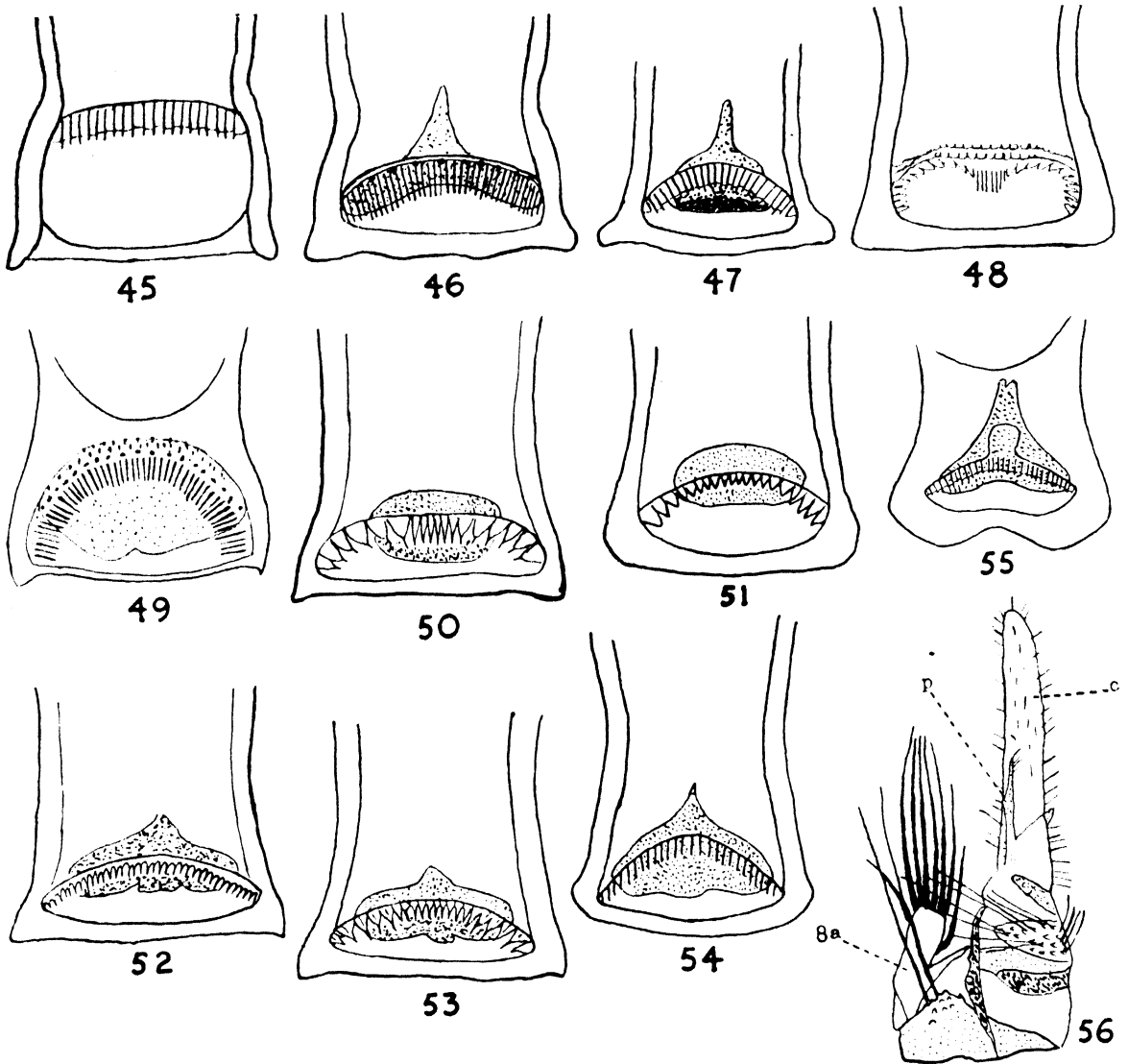


FIG. 45. Buccal armature of *P. simillimus* (female).

FIG. 46. " " *P. africanus* (female).

FIG. 47. " " *P. signatipennis* (female).

FIG. 48. " " *P. ingrami* (female).

FIG. 49. " " *P. serratus* (female).

FIG. 50. " " *P. schwetzi* (female).

FIG. 51. " " *P. schoutedeni* (female).

FIG. 52. " " *P. congolensis* (female).

FIG. 53. " " *P. congolensis* var. *distinctus* (female).

FIG. 54. " " *P. yusafi* (female).

FIG. 55. " " *P. babu* (female).

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Med. Res., vol. 20, p. 73.)

FIG. 56. *P. transvaalensis*, terminalia of female; c = cerci, p = post-genital plate, 8a = eighth abdominal sternite. (Reproduced, by kind permission, from the *Indian Jl. Med. Res.*, vol. 20, p. 880.)

THE EXPERIMENTAL INFECTION OF RATS WITH *ENTAMOEBIA HISTOLYTICA*; WITH A METHOD FOR EVALUATING THE ANTI-AMOEBIIC PROPERTIES OF NEW COMPOUNDS

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The unsatisfactory results of treatment of human amoebiasis discussed in recent reviews by Manson-Bahr (1944), Adams (1945), Lamb and Royston (1945) and Hargreaves (1945) have emphasized the need for a new and more efficient anti-amoebic drug. Unfortunately, no method has hitherto been available for the large-scale routine testing of possibly amoebicidal compounds *in vivo*, and this has undoubtedly limited progress in the chemotherapy of the disease. An experimental amoebic infection can be produced in cats, dogs or monkeys, with which animals a limited amount of experimental chemotherapeutic work has been carried out. For obvious reasons, however, they are not suitable for extensive investigations of new compounds, and clearly the need is for a method of producing a suitable amoebic infection in a smaller laboratory animal conveniently obtainable in large numbers. This need is emphasized by the fact that, although a method for *in vitro* testing is available, viz., that of Laidlaw, Dobell and Bishop (1928), its application by Pyman (1937) led to the production of a compound which, although highly active *in vitro*, was of no use clinically.

Many workers have sought to produce in rats an experimental infection comparable to the disease in man, but with little success. Chiang (1925), Atchley (1936) and Tsuchiya (1939) were able to produce only a low incidence of infections of low grade, but Böe (1939) claimed a high incidence of low-grade infections, and Lynch (1915) and Kessel (1923) both recorded ulceration in isolated instances. These diverse results suggested that it would be of advantage to reinvestigate the problem.

This paper records methods for producing in rats an experimental amoebic infection which can be used for chemotherapeutic investigations.

EXPERIMENTAL WORK

In most of the experimental work described below, cultures of *Entamoeba histolytica* were used. The following simple liquid medium was employed, which is a modification of the medium of Pavlova (1938) and has the following composition:

Sterile horse serum	0.5 c.cm.
1 per cent. marmite* solution	1.0 c.cm.
'Buffer saline solution' (pH 7.2)	8.5 c.cm.
Rice starch	30 mgm.

The 'buffer saline solution' (pH 7.2) has the following composition:

Disodium hydrogen phosphate, Na_2HPO_4 (9.476 gm./l.)	375 c.cm.
Potassium dihydrogen phosphate, KH_2PO_4 (9.078 gm./l.)	125 c.cm.
Sodium chloride (0.9 per cent. solution)	2,250 c.cm.

* Marmite is an autolysed yeast extract made by Marmite Limited, London.



FIG. 1. Large intestine of rat experimentally infected with *E. histolytica*: shallow ulceration; $\times 40$.



FIG. 2. Same as fig. 1; $\times 140$.



FIG. 3. Large intestine of rat experimentally infected with *E. histolytica*: deep perforating ulcer; $\times 40$.



FIG. 4. Same as fig. 3; $\times 140$.



FIG. 5. Large intestine of rat experimentally infected with *E. histolytica*: shallow ulceration; $\times 85$.



FIG. 6. Same as fig. 5; $\times 450$.



FIG. 7. 1, 2 and 4: caecums of heavily infected rats; note inflammation in the ileo-caecal region in 1, 2 and 4, and the ulceration in the apex of 4; ulceration was present in 1 and 2, but is not clearly shown in the photograph. 3: caecum of a normal rat.

For maintaining stock cultures of *E. histolytica*, 10 c.cm. of medium contained in 6 in. \times $\frac{5}{8}$ in. tubes was used, and for growing the amoebae on a large scale 300 c.cm. of medium contained in Roux bottles.

INJECTION OF CULTURES OF *E. HISTOLYTICA* INTO RATS

In some preliminary experiments, cultures of *E. histolytica* were injected rectally, or intracaecally after laparotomy, into three-months-old rats. Subsequently, no signs of amoebic infection were detected in any of the rats, either by daily examination of the faeces or at autopsy about a fortnight after injection. Cultures made of the rat faeces also were negative for *E. histolytica*. Altogether 46 rats were used, and auxiliary means employed in attempts to aid the production of an infection included preliminary starvation, purging with magnesium sulphate, modification of the diet, and occlusion of the anus with a collodion pad.

INJECTION OF INFECTED FAECAL MATERIAL FROM KITTENS INTO RATS

It was thought that better results might be obtained by using fresh dysenteric stools, e.g., from an experimentally infected kitten. After many unsuccessful attempts, an infection was produced in a kitten by the rectal injection of a culture of *E. histolytica*, and, once established, was easily maintained by passage. A ready supply of dysenteric material was thus made available. The method used was similar to that described by Dale and Dobell (1917).

The results obtained from injection of this dysenteric material into young rats were encouraging. Not only were the rats parasitized with *E. histolytica*, but at autopsy some of them showed marked inflammation and even ulceration in the caecum. In addition, stained sections demonstrated beyond doubt the presence of considerable numbers of *E. histolytica* in the tissues of the gut (see Plates IV and V, figs. 1-6).

In these preliminary experiments four-weeks-old rats (weighing 20-35 gm.) were injected, either intracaecally after laparotomy or rectally, with a suspension in saline of freshly passed dysenteric faecal material. In the 'intracaecal group' each of eight rats was found to be infected with *E. histolytica*. Definite amoebic ulceration was seen at autopsy (after 11-14 days) in the case of six rats, while a seventh, although showing no gross signs of infection in the gut, suffered a light infection, as shown by microscopic examination of a smear made from the gut wall. The remaining rat, which was allowed to survive, became free from the infection after eight weeks, although in the early stages it had been passing *E. histolytica* in the faeces. In the 'rectal group' two out of 11 rats had amoebic ulceration in the caecum when killed and examined after 28 days. The remainder were apparently normal.

Later experiments confirmed that the results obtained after intracaecal injection were very much better than those after intrarectal, so that, although involving an operative procedure, the former method was definitely preferable. The variation in degree of infection of different animals noticed in the original experiments was again experienced. In some rats the caecum was markedly inflamed, hyperaemic and thickened, or even ulcerated, whereas in others there was no obvious sign of infection, although in smears amoebae were present. For convenience in recording these results, six arbitrary degrees of infection were chosen. They are explained in Table I.

TABLE I
The degree of amoebic infection found in experimentally infected rats

Symbol	Pathological description
0	Gut apparently normal ; no amoebae detectable in smear.
1	Gut apparently normal ; small number of amoebae in smear (1-20 in whole preparation).
2	Gut apparently normal ; moderate number of amoebae in smear (20-100 in whole preparation).
3	Gut slightly inflamed (slight hyperaemia or thickening) ; numerous amoebae in smear.
4	Marked inflammation ; much mucus present, numerous amoebae in smear.
5	Inflammation and ulceration ; numerous amoebae in smear.

Using this scheme of assessment, an 'average degree of infection' (A.D.I.) can be calculated for a group simply by calculating the arithmetic average of the assigned symbols. The A.D.I. is thus a means of comparing one group with another.

Despite the subjective nature of the assessment, it was found that two independent observers showed remarkably close agreement in a series of experiments (Table II).

TABLE II
Values obtained for A.D.I. of 22 groups by two observers

Obs- erver	Average degree of infection of groups																					
A	3.4	2.6	1.3	1.6	2.5	2.6	2.0	0.2	4.5	3.8	3.8	2.6	3.3	2.1	2.5	2.4	2.6	1.7	2.8	2.1	2.5	3.8
B	2.8	2.8	1.4	1.6	2.7	2.4	2.1	0.1	4.5	3.3	3.5	2.2	3.2	2.0	2.5	2.3	2.5	1.7	2.8	2.1	2.7	3.5

INFLUENCE OF THE WEIGHT OF THE RATS ON THE EXPERIMENTAL INFECTION

Evidence from early experiments suggested that the weight of the rats was important in determining the infection. To investigate this point, the results obtained with rats of

TABLE III
The average degree of infection of rats in various weight-groups

No. of rats in group	Weight, in gm.	Average degree of infection
38	18-20	3.12
39	21-23	3.10
76	24-26	2.67
77	27-29	2.54
93	30-32	2.35
84	33-35	2.32
75	36-38	1.70
69	39-41	1.55
29	42-44	1.21
29	45-47	1.03
37	48-52	0.65
30	53-60	0.86
24	61-80	0.71

similar weight were abstracted from a large number of experiments and grouped together. An A.D.I. for each weight-group was determined. The results showed that there was a definite falling off in A.D.I. with increase in body-weight (Table III), and this relationship is clearly shown in the graph (fig. 1).

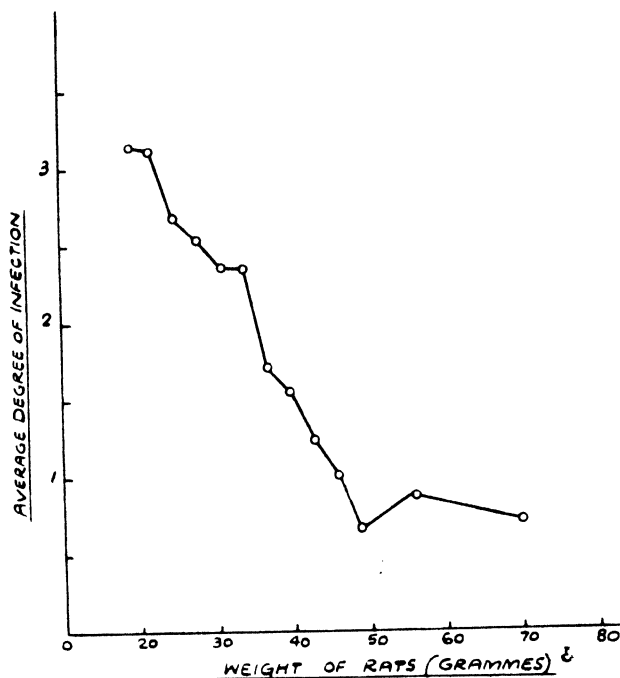


FIG. 1. Average degree of infection of rats in various weight-groups.

OTHER INFLUENCES ON THE EXPERIMENTAL INFECTION

Other factors whose effects on the infection have been studied are the following :

1. Deliberately inflicted trauma in the caecum.
2. Diet modification, e.g., high carbohydrate diet, high protein diet, liver diet.
3. Site of injection (intra-ileal, intracolonic, rectal, submucosal).
4. Preliminary purging with magnesium sulphate, castor oil, or various vegetable cathartics, particularly those acting specifically in the large intestine.
5. Simultaneous injection of bacterial cultures, e.g., *Ps. pyocyanea*, *Str. pyogenes*.

Apart from preliminary purging with castor oil, none of the above modifications produced marked improvement in the average degree of infection. Castor-oil treatment tended to increase the A.D.I., but this treatment, combined with the operative procedure, caused an increase in post-operative mortality.

By using rats weighing 20-33 gm. (3-4 weeks old) a reasonably standard control-infection could be achieved, with an A.D.I. usually of about 2.5-3.5. It was considered that this would be adequate for use in chemotherapeutic experiments. Some indication of the degree of uniformity of control-groups can be obtained from a study of the 20 consecutive experiments recorded in Table IV.

TABLE IV

Control-groups in 20 consecutive experiments, November, 1944, to February, 1945

Experiment no. ...	131	132	133	134	136	137	138	139	140	141	142	143	144	145
Average weight of rats ...	27	25	29	28	25	30	26	26	30	28	27	28	29	32
No. infected ...	9/12	7/10	8/10	8/9	6/8	11/14	7/8	10/10	9/11	10/10	9/11	10/10	9/12	9/11
A.D.I. ...	2.3	3.3	2.2	3.1	3.4	2.9	4.0	3.8	2.9	4.3	2.3	4.7	2.8	2.5

Experiment no. ...	146	147	148	149	150	151
Average weight of rats ...	33	29	27	28	30	31
No. infected ...	8/11	5/7	9/12	6/11	8/10	6/9
A.D.I. ...	3.0	3.5	3.1	2.8	3.3	2.3

INJECTION OF CULTURES OF *E. HISTOLYTICA* SUSPENDED IN MUCIN

By using concentrated cultures of *E. histolytica* mixed with an equal volume of a 10 per cent. suspension of mucin, it was found possible to produce results as good as those obtained when the inoculum was prepared from the dysenteric stools of an infected kitten. It is probable that the poor results obtained previously with cultures of *E. histolytica* were due not only to the unsuitable weight of the rats, but also to the physical form of the inoculum. Of several types of mucin which were tried, only that supplied by Wilson and Company Incorporated, Chicago, U.S.A. (type 1701-W) was found to produce a suitably sticky suspension, and this has been used in all subsequent experiments.

COURSE OF THE INFECTION IN RATS

To establish the normal course of the untreated infection, several groups, each containing 60 rats (weighing 20-33 gm.), were infected. From each group a proportion were killed and examined after certain intervals of time, and the results were grouped together. They are recorded in Table V. Fig. 2 shows graphically the course of the infection, and the following additional facts may be stated:

1. Ulceration may be detected as early as 24 hours after the operative injection.
2. The infection is at its height 3-6 days after the operation.
3. The infection may gradually disappear completely from a majority of rats during the period 7-28 days after the operation.
4. Cysts of *E. histolytica* may occasionally be detected, usually after 14-28 days.
5. Only a small proportion of the rats show obvious clinical signs of infection, e.g., diarrhoea with mucus and blood. Some rats showing caecal ulceration on a massive scale at autopsy appear normal during life.
6. The infection is not reliably detected by a study of the faeces (direct microscopic examination or culturing).

TABLE V

Course of the experimental amoebic infection in rats
(a) 1-7 days after operation

Experiment	Average degree of infection after (days)						
	1	2	3	4	5	6	7
1	2.5	3.7	3.4	5.0	—	—	3.1
2	1.4	3.9	4.3	4.0	4.7	—	—
3	2.3	2.4	3.1	—	2.5	—	3.7
4	3.1	3.0	—	2.6	4.1	2.7	—
5	—	3.6	2.7	3.6	3.6	—	2.1
6	1.8	—	2.3	—	1.8	4.3	3.6
7	—	2.8	4.9	3.8	4.4	—	4.5
8	3.4	—	4.5	—	4.3	3.0	2.6
Total rats	60	68	66	56	62	30	59
Group A.D.I.	2.33	3.24	3.47	3.80	3.61	3.66	3.30

(b) 3-27 days after operation

Experiment	Average degree of infection after (days)				
	3	6	13	20	27
1	4.7	4.0	3.7	2.4	1.4
2	—	2.8	2.8	1.1	0.8
3	4.1	4.3	3.1	1.1	0.7
4	4.1	4.7	3.4	1.7	1.2
5	3.6	2.8	1.4	0.5	0.5
6	2.6	1.9	2.0	1.5	2.7
7	3.7	3.5	3.6	1.2	1.8
8	4.0	2.7	0.3	1.3	1.1
Total rats	67	78	71	70	69
Group A.D.I.	3.83	3.31	2.59	1.37	1.21

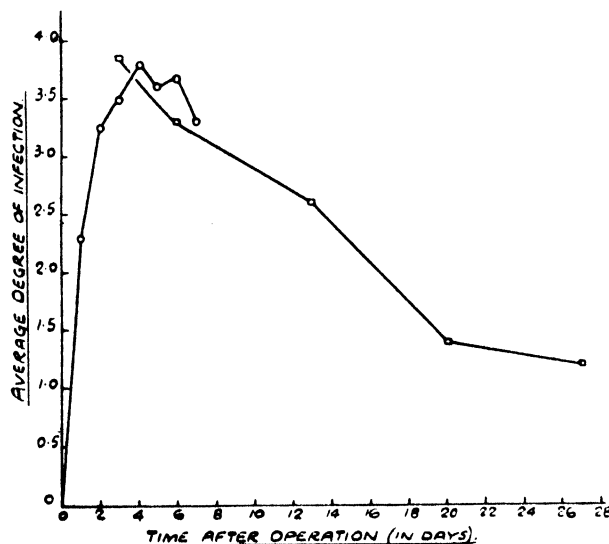


FIG. 2. Course of experimental amoebic infection in rats.

THE USE OF THE EXPERIMENTAL INFECTION IN THERAPEUTIC STUDIES

Subcutaneous or oral injections of 1-2 mgm./kgm. emetine hydrochloride made at the time of the operation, and on the five subsequent days, caused a marked lowering of the A.D.I. A single dose of the drug (12 mgm./kgm.) given 24 hours after the operation produced a similar effect. Other compounds used clinically in the treatment of amoebiasis were examined in a similar manner, and a standardized procedure for examining new compounds has now been adopted. It is as follows.

Sixty rats, aged 3-4 weeks and weighing 20-33 gm., are divided according to weight into five groups of 12 rats. A concentrated suspension of *E. histolytica* is prepared by centrifuging the combined deposits from 12 Roux bottle cultures grown for two days at 37° C. in the medium described above (300 c.cm. per bottle). The volume is reduced to 8 c.cm., and then the suspension is mixed with an equal volume of 10 per cent. mucin in water ground to a smooth cream. The mixture is kept in a water-bath at 37° C. and contains approximately 1,000,000 amoebae per c.cm. The rats are anaesthetized with ether and injected intracaecally after laparotomy with 0.2 c.cm. of the above suspension. Strict aseptic procedure is unnecessary. A good average rate of carrying out the operations with the aid of an assistant is 50-55 rats per hour.

One group is left untreated to serve as a control. The remaining groups are dosed with the drug under test 24 hours after the operation.

The rats are killed six days after the operation, and a careful examination of the caecum is made as follows:

1. After excision the caecum is cut open and any faecal matter present is carefully scraped away with a small broad spatula.
2. The macroscopic appearance of the caecum is noted.
3. A smear is made after scraping over all the mucosal surface with a stout wire loop.
4. The smear is examined under the microscope and the approximate concentration of *E. histolytica* is noted.

An assessment of the degree of infection of each rat is made as described above (see Table I), and an A.D.I. is calculated for each group.

ASSESSMENT OF RESULTS

The following statistical formula is used to assess the significance of the difference between the A.D.I. of a treated group and that of a control:

$$z = \frac{x - y}{\sqrt{\frac{\sigma^2 x}{m} + \frac{\sigma^2 y}{n}}}$$

where x and y = the A.D.I. for the control and treated groups respectively, m and n = the numbers of rats in the control and treated groups respectively, and σx and σy = the standard deviations for x and y respectively. The values for z for a few selected levels of significance are:

$P = 0.2$	$z = 1.28$
$P = 0.1$	$z = 1.64$
$P = 0.05$	$z = 1.96$
$P = 0.01$	$z = 2.58$

A fuller range is given by Fisher and Yates (1938).

The values of σ_x and σ_y used in the above formula are read from a curve derived from the results of a large number of control and treated groups, representing the results obtained over a period of two years. The standard deviation of the variation of degree of infection between rats was plotted against the A.D.I. for each group, and a mean curve representing this relationship was drawn (fig. 3). The variation of the individual standard deviations about this curve may be satisfactorily accounted for by random sampling errors. Furthermore, no trend was apparent over the period, and there was no evidence that the variability between rats differed for experiments carried out at different times. The curve in fig. 3 may therefore be taken as representing the standard deviation for any given A.D.I.

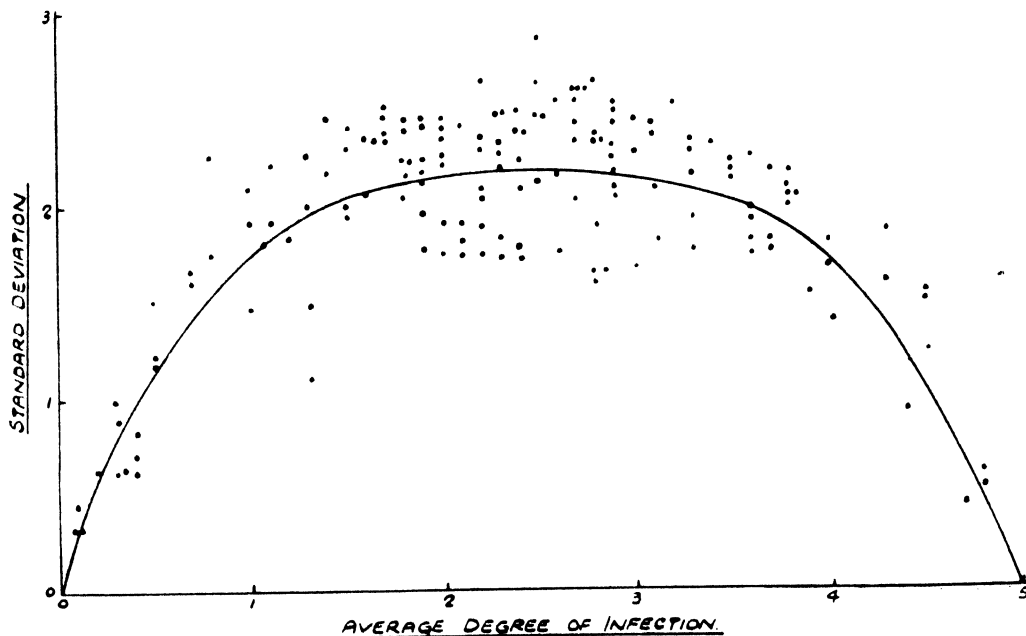


FIG. 3. Graph correlating standard deviation and average degree of infection.

Examples of therapeutic results obtained with a variety of compounds, some of which are used clinically in the treatment of amoebiasis, are recorded in Table VI.

In Table VI compounds giving a P value of 0.05 or less are regarded as active. Where the P value is 0.05–0.20, slight activity is indicated, but anything greater than 0.20 suggests activity of a low order. Thus, of the compounds dealt with in the table, chiniofon, carbarsone and mepacrine show activity at high doses, and emetine, stovarsol and penicillin show slight activity. The remainder of the compounds appear to be inactive.

The interesting observation has been made that infections produced by different 'strains' of *E. histolytica* respond differently to treatment with emetine hydrochloride, as shown in Table VII.

TABLE VI

The therapeutic action of selected compounds against experimental amoebiasis in the rat

Compound	Dose (mgm./ kgm.)	No. infected		A.D.I.			z	P
		Treated	Untreated	Treated	Untreated	Difference		
Emetine HCl ...	15	7/12	15/19	1.25	2.3	1.05	1.435	0.15
Chiniofon ...	1,000	0/12	8/12	0	3.1	3.1	5.043	<0.01
Chiniofon ...	300	4/12	8/12	1.65	3.1	1.45	1.696	0.09
Chiniofon ...	100	10/14	8/12	3.3	3.1	0.2	0.2417	0.8
Carbarsone ...	1,000	7/12	12/12	2.0	3.85	1.85	2.302	0.02
Stovarsol ...	1,000	2/10	7/11	0.8	1.9	1.1	1.387	0.17
Rivanol ...	1,000	10/11	7/11	2.35	1.9	0.45	0.4881	0.63
Sulphaguanidine	1,000	5/10	7/12	2.0	2.5	0.5	0.5274	0.59
Sulphamezathine	1,000	8/10	7/12	2.6	2.5	0.1	0.1046	0.92
Mepacrine HCl ...	1,000	4/12	12/12	1.35	3.35	2.0	2.477	0.02
Phenothiazine ...	400	5/10	8/10	1.7	2.2	0.5	0.5242	0.59
Penicillin ...	*	3/19	5/15	0.42	1.47	1.05	1.886	0.06
'D.D.T.' ...	100	6/10	10/12	2.5	3.0	0.5	0.535	0.59

* Penicillin dosage was 500 units given subcutaneously after 24 hours and every three hours thereafter until a total of 4,000 units had been given.

TABLE VII

The effect of emetine hydrochloride on infections produced in rats by three strains of *E. histolytica*

Strain	Date	Dose of emetine HCl	No. infected		A.D.I.			z	P
			Treated	Un- treated	Treated	Un- treated	Diff- erence		
Whatman	23.3.43	6 × 2 mgm./kgm. s.c.	7/24	20/24	0.79	3.12	2.33	4.43	0.01
Whatman	4.5.44	1 × 12 mgm./kgm. p.o.	2/11	6/9	0.45	2.66	2.21	2.79	0.01
Clark	21.7.44	1 × 12 mgm./kgm. p.o.	5/10	7/9	2.1	3.37	1.27	1.31	0.19
Walton	21.7.44	1 × 12 mgm./kgm. p.o.	7/11	8/12	2.45	2.50	0.05	0.05	0.96
Walton	11.8.44	1 × 15 mgm./kgm. p.o.	7/12	15/19	1.25	2.31	1.06	1.45	0.15

DISCUSSION

There are probably two main reasons why other workers failed to find the rat a suitable animal for the study of experimental amoebiasis. The first is that mature rats were usually used, and the second is that close observation of infected animals was not usually made during the acute phase of the infection. The six-weeks-old rats which Atchley used in some of his experiments would probably have weighed 60–80 gm., and it is not surprising that he found only light infections. Where the age or weight of the rats was recorded in other experiments it was similarly unfavourable. The transient nature of the infection and the absence of any gross external signs of disease, e.g., diarrhoea, mucus, or numerous amoebae in the faeces, also have been misleading in the past. It is likely that a combination of these two important factors has been responsible for the variable results obtained by others, and more rigid control of these factors in the future should give better results.

In using the experimental infection for chemotherapeutic tests it is likely that the effectiveness of most compounds will be demonstrated by a single dose given 24 hours after the operation. Where compounds are known to be excreted rapidly, however, it would be unwise to rely upon this method of dosing, and a more extended course should be given in such cases. For instance, the slight activity of penicillin was only demonstrated by giving large doses every three hours. A single dose was ineffective.

Although the test is one of the action of compounds against an acute amoebic infection in the rat, drugs which are useful in chronic amoebiasis in man show up favourably, e.g., chiniofon, carbarsone, stovarsol. There is thus every hope that new compounds may be found by this test which will prove of use in human amoebiasis.

The different response to treatment with emetine of the infections produced by three strains of *E. histolytica* calls for comment, but so far insufficient evidence is available to warrant any conclusions regarding the possibility of 'emetine-resistant strains.' It is clear, however, that the method affords a useful means of investigating the problem.

Regarding the statistical treatment of the results, it is probable that the curve shown in fig. 3 may not hold for different laboratories or following changes in technique or strain of rats. In such cases, and until the accumulation of sufficient experimental data, the significance of a treatment-effect may be estimated by the *t*-test (Fisher, 1938), calculating the standard deviation from the combined variation within the groups for A.D.I.s between 1 and 4. The range may be extended to include virtually all A.D.I.s, by the use of the sine transformation (Fisher and Yates, 1938; Table XII).

When the technique has been standardized, and when sufficient data are available for the construction of a graph, as in fig. 3, the use of the graph introduces considerable simplification in the assessment of the significance of a treatment-effect. It is, however, necessary to check the validity of the graph from time to time.

If significant changes are apparent in the standard deviation in experiments carried out at different times, the full *t*-test should be used, with each experiment supplying its own estimate of the standard deviation.

In the analysis of the results recorded in Table VI, the probability-levels of 0.05 and 0.20 were selected as indicating levels of significant activity and slight activity respectively. The probability-level used in interpretation of chemotherapeutic results will, of course, depend upon whether one is searching merely for slight indications of activity to provide clues for further work or for a compound worthy of further investigation in other animals. It is probable, judging from the results obtained so far, that a *P* value of 0.20 is adequate for the former purpose, whereas 0.05 is necessary for the latter. Clearly the *P* values can be used to interpret a grading of amoebicidal action between the extremes of complete sterilization of an infection and complete inactivity.

SUMMARY

1. Attempts to infect three-months-old rats using cultures of *Entamoeba histolytica* injected rectally or intracaecally failed to produce any infections.
2. Using freshly passed faecal material from a kitten experimentally infected with *E. histolytica*, amoebic infections were produced in rats 3-4 weeks old. Gross amoebic ulceration was observed. Cultures of *E. histolytica* suspended in 5 per cent. mucin gave similar results.
3. Variation in degree of infection among groups of experimentally infected rats

was usual, and attempts to limit this variation are described. The two main factors which influenced the average degree of infection of a group were (a) the weight of the rat, and (b) the time after the operation at which the post-mortem examination was made. Rats weighing 20–33 gm. gave the best results. The infection was maximal after 3–6 days.

4. Cysts of *E. histolytica* were observed on rare occasions.

5. A technique for examining the therapeutic action of speculative compounds against experimental amoebiasis in the rat is described in detail.

6. Results of the therapeutic action of selected compounds are recorded.

ACKNOWLEDGEMENTS.—It is a pleasure to acknowledge the help of Dr. O. L. Davies, who formulated the statistical method of interpreting the results. I am also indebted to Mr. E. Young, who is responsible for the photomicrographs.

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SALIVATION BY *GLOSSINA MORSITANS* ON TO GLASS SLIDES: A TECHNIQUE FOR ISOLATING INFECTED FLIES

BY

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Bruce *et al.* (1914, 1915) induced infected flies to salivate on to cover-slips. The smears of saliva were immediately fixed and stained with Giemsa's stain. In this way they obtained preparations of both metacyclic and/or other developmental forms of *Trypanosoma simiae*, *T. brucei* vel *rhodesiense*, *T. pecorum* and *T. caprae*. No record is known to the writer of this technique having been used by any other worker.

While the infectivity to man of *T. rhodesiense* was being investigated at Tinde laboratory, the need was felt for information regarding the numbers of trypanosomes which an infected fly could eject when it fed. For this purpose the writer employed a new method, which is a modification of the technique evolved by Bruce and his collaborators. This method, which was briefly described elsewhere (Burtt and Fairbairn, 1945), is as follows.

Slides are lightly smeared with albumen (taken direct from an egg) and allowed to dry. The fly to be studied is contained in a small bottle, having a mouth about $1\frac{1}{4}$ inches wide, closed with mosquito-netting. First the fly is tested, to find if it wishes to probe, by holding a guinea-pig near it. The bottle with the hungry fly is clamped, mouth downwards, in a burette-stand, and a guinea-pig is brought close up to the mosquito-netting in order to stimulate probing. As the fly attempts to bite, the guinea-pig is withdrawn slightly, so that the hungry fly protrudes its proboscis down through the netting in its attempt to reach the animal. One of the albumen slides is interposed, so as to come into contact with the tip of the fly's proboscis. The guinea-pig is then removed and the fly continues to probe on the surface of the slide, its proboscis usually executing intricate agitated movements during the process. When the fly retracts its proboscis (usually after about 30 seconds) the slide is inspected by reflected light, to confirm the presence of salivary tracks. It is then allowed to dry and is fixed for fully two minutes with absolute alcohol, after which it is dried in the air and stained with Giemsa's stain.

From several hundred such tests it was found that the technique was reliable for detecting flies known to be infected with *T. rhodesiense*. It was decided to see whether it would also prove effective for the isolation of all infected flies in transmission-experiments. Dissection of flies had proved unreliable (Burtt, 1946), so that all infection-rates had to be based on results obtained by feeding individual flies on separate clean white rats. This necessitated awaiting the completion of the incubation-period of the infection in the animal before a positive fly could be detected, and was also very laborious.

Accordingly, the two methods of examination were tested in parallel. A probe record of the hungry fly was first taken on an albumen-smeared slide, and then the fly was immediately fed on a rat. Flies detected by the slide method were only counted as positive after they had been proved infective to a clean animal. The results obtained by the two methods are shown in Table I.

TABLE I

The number of infected flies (*G. morsitans*) detected respectively by a single test on rats and a single test on albumen-smear slides

No. of flies examined	Positive flies detected			
	Total	By both methods	By slide only	By rats only
2,716	183	142	17	24

Table I shows that each method failed to detect a proportion of infected flies, and that rats were somewhat more reliable than slides. Both categories of tests were applied only once. It was evident, in the case of rats, that only those flies in which the trypanosome-cycle had been completed could prove infective and be detected. Failure in the case of slides seemed to be due to the infection in the salivary glands being still too light to render detection practicable.

Accordingly, another set of tests was done in parallel. This time, after the first probe and feed on a rat, the flies were re-examined about 10 days later by a second probe test. The results are shown in Table II.

TABLE II

The number of infected flies (*G. morsitans*) detected respectively by a single test on rats and two tests on albumen-smear slides done at an interval of approximately 10 days

No. of flies examined	Positive flies detected			
	Total	By both methods	By slides only	By rats only
558	27	24	3	Nil

Table II shows that every infected fly was thus detected by the slide technique.

In 4,101 flies tested, 46 extruded only proventricular forms when first detected. Of these, 17 eventually became infective. This pointed to the conclusion that the full number of salivary-gland-infected flies in any transmission-experiment could be found if probe tests were done over a period of about 10 days, every time the fly was hungry.

It was found that the best results with probes were obtained with the use of albumen, whereas serum was unreliable. When probing was done into a drop of fluid (serum, defibrinated blood or Ringer-glucose serum) in order to recover living trypanosomes, difficulty was experienced in getting any considerable number. The best chance of success was to stimulate the hungry fly for a considerable period with the guinea-pig held near it, before getting it to probe into the fluid.

The probe method was satisfactory for isolating flies infected with *T. rhodesiense*. *T. simiae* was also isolated in this way recently at Tinde, but in most cases the infected flies had to be tested repeatedly before they could be detected and trypanosomes were extruded in very small numbers.

FEATURES SHOWN BY THE TECHNIQUE

1. Viewed by reflected light the saliva tracks appear dull and opalescent. A fly did not always extrude saliva during a single probe, but failure to do so was not frequent.

Only two instances were encountered when a fly failed to salivate on three consecutive probes, and in both cases the fly concerned had died by the following day.

2. Although some salivary record can usually be obtained on the slide without the use of albumen, the presence of the latter makes a great improvement to the preparation. Without it, the salivary record is tattered and the staining inferior. If the saliva remains colourless after staining it is extremely difficult to find it on the slide. When albumen is used every trace deposited by the fly is apparently retained, as shown by the boundaries of the tracks being smooth and entire. The albumen stains blue and thus gives a contrast with the saliva, which is colourless, purplish or pinkish-red. Usually there is a deeper-staining border at the edge of the saliva track which accentuates the contrast between it and the albumen. Where the proboscis breaks contact with the slide, the record tails off into deeply staining points. A typical salivation is shown in Plate VI, fig. 1 ($\times 20$).

The stained saliva shows various features. A deeply staining substance which takes the form of threads, tufts or bunches is usually in evidence (Plate VI, fig. 2; $\times 600$). Frequently the deeply staining substance is present in dots aggregated together, as shown in Plate VI, fig. 4 ($\times 300$). Sometimes it appears as if crystallization had taken place, leading to a feathery structure (Plate VI, fig. 5; $\times 250$). More rarely, minute bubbles are found in the saliva track, or the saliva becomes folded on itself (Plate VI, fig. 3; $\times 75$). Sometimes blood is regurgitated (Plate VI, fig. 6; $\times 60$).

3. Usually in flies in which the trypanosome infection had been long established the saliva remains colourless after staining, save for a bluish-grey border at its edge. This is shown in Plate VII, figs. 7 ($\times 40$) and 8 ($\times 300$).

4. Figs. 7, 8, 9 and 10 in Plate VII show metacyclic trypanosomes at different magnifications ($\times 40$, 300, 800, 1,200) in sample preparations of *T. rhodesiense*, obtained by this technique.

5. Sometimes inclusions in the form of a small black dots, of unknown nature, are encountered.

6. Bacteria were observed in the saliva of 26 out of 3,856 flies. Plate VII, fig. 9 ($\times 800$), shows an example of this. There were various types in different flies, some tending to form chains, others rounded and attached in pairs. All the flies mentioned above had been fed on animals infected with *T. rhodesiense* and belonging to various species. They were distributed as shown in Table III.

TABLE III
The incidence of bacteria in the saliva of *G. morsitans*

Trypanosomes	Bacteria		Total
	Present	Absent	
Present	13	233	246
Absent	13	3,597	3,610
Total	26	3,830	3,856

A significantly higher proportion ($P < 0.01$) of bacteria were found in flies infected with trypanosomes. They persisted throughout the fly's life and their presence seemed to

exert no harmful effect on the insect, or on the trypanosomes if these happened also to be present. Infected flies, with bacteria, were tested on man on many occasions. The presence of bacteria produced no abnormal results, showing that they were non-pathogenic.

SUMMARY

1. A simple technique is described for making preparations of the salivary forms of *Trypanosoma rhodesiense* from living infected *Glossina morsitans*.

2. The technique proved effective for detecting flies known to be infected with *T. rhodesiense*, and also for isolating all the infected flies occurring in transmission-experiments.

3. In 4,101 flies tested, 46 extruded only proventricular forms when first detected. Of these, 17 eventually became infective.

4. It was found that the best results with probes were obtained with the use of albumen, whereas serum was unreliable. To recover living trypanosomes, the best chance of success was to stimulate the hungry fly for a considerable period with a guinea-pig held near it, before getting it to probe into a drop of fluid.

5. Flies infected with *T. simiae* were also isolated by means of the probe technique, but usually only after repeated tests had been made.

6. Features shown by the technique are described and illustrated.

7. Bacteria were observed in 26 out of 3,856 flies. A significantly higher proportion ($P < 0.01$) of bacteria were found in flies infected with trypanosomes. Some of the latter, with bacteria, were tested on man, but no abnormal results were produced.

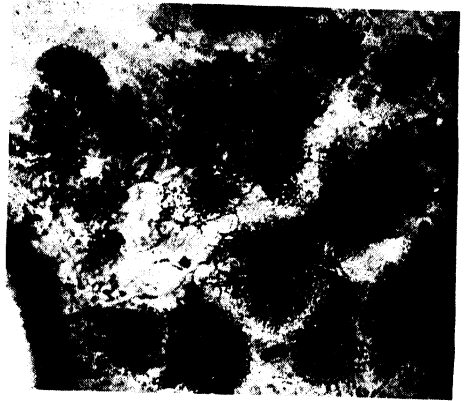
ACKNOWLEDGEMENTS.—The writer tenders his thanks to the Acting Director of the Wellcome Laboratories of Tropical Medicine, London, for placing at his disposal the facilities for making the illustrations, and to Mr. A. A. Michieli for taking the photomicrographs; also to Dr. H. Fairbairn, Dr. C. A. Hoare and Dr. C. H. N. Jackson for helpful advice and criticism, and to the last-named for doing the statistical analysis.

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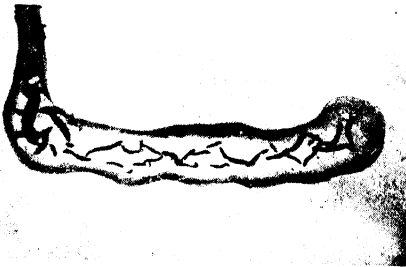
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STUDIES ON SYNTHETIC ANTIMALARIAL DRUGS

XVI.—THE ABSORPTION, DISTRIBUTION AND EXCRETION OF 2-*p*-CHLOROPHENYLGUANIDINO-4- β -DIETHYLAMINOETHYL- AMINO-6-METHYLPYRIMIDINE (3349) IN EXPERIMENTAL ANIMALS

BY

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INTRODUCTION

It has already been shown (Spinks and Tottey, 1945*b*) that the excretion of 3349 in human urine closely resembles that of mepacrine. This study was undertaken to provide more complete information, particularly as regards those aspects of the behaviour of 3349 that might throw light on the mode of its antimalarial action. All experiments were, therefore, based on the known properties of mepacrine and quinine, and have been standardized so as to facilitate comparison of 3349 with them and with new drugs now being prepared in the laboratories of Imperial Chemical Industries Limited. Since some of these new drugs were available only in small amount, the rat and mouse were chosen as experimental animals; they were found to be adequately similar to man. 3349 was determined throughout by the colorimetric method (Spinks and Tottey, 1945*a*), unless otherwise stated.

EXPERIMENTAL SECTION

Absorption and Distribution of 3349 Following the Oral Administration of 100 mgm. of base/kgm. in the Rat

Groups of three albino rats (180–250 gm.) were given 100 mgm. of base/kgm. by stomach-tube as a 1 per cent. solution of the dihydrochloride, and were sacrificed at intervals after dosing by withdrawal of heart blood under chloroform anaesthesia. Concentrations of 3349 in pooled blood, plasma, liver, lung, spleen and kidney are shown in diagrams 1 and 2. Diagram 1 also gives a plasma curve of quinine administered under the same conditions and in the same dose. Quinine was determined by a new method, details of which are given at the end of this paper. Two analyses of brain were also made, using the turbidimetric method (Spinks, 1945). Concentrations therein after half an hour and 24 hours were 1.18 and 18.1 mgm./kgm. respectively.

Distribution of 3349 Following the Intravenous Administration of 25 mgm. of base/kgm. in the Rat

Groups of three rats received 25 mgm./kgm. intravenously and were sacrificed at intervals. Concentrations in pooled tissues are shown in diagram 3.

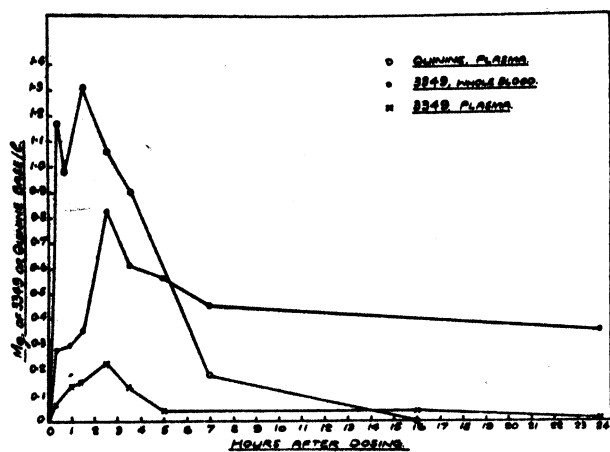


DIAGRAM 1. Blood and plasma concentrations of 3349 and quinine in the rat following the oral administration of 100 mgm. of base/kgm.

DIAGRAM 2. Tissue concentrations of 3349 in the rat following the oral administration of 100 mgm. of base/kgm.

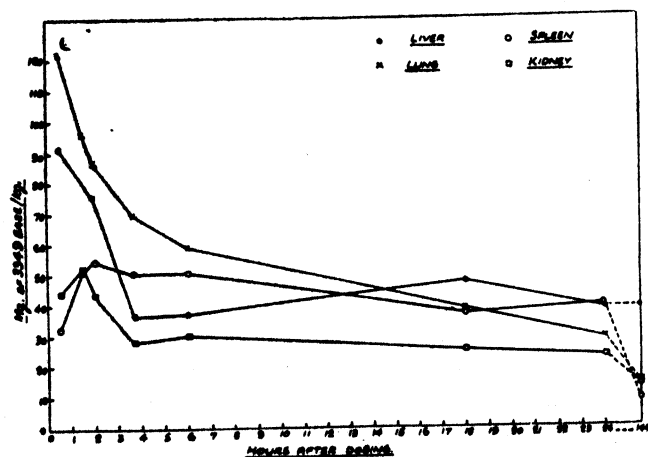
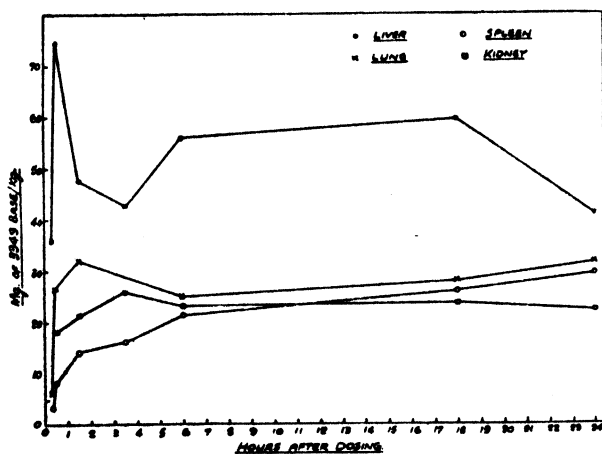


DIAGRAM 3. Tissue concentrations of 3349 in the rat following the intravenous administration of 25 mgm. of base/kgm.

Blood concentrations were 1.82, 1.32, 0.54, 0.74, 0.27 and 0.26 mgm./l. after 0.30, 1.30, 3.40, 6.00, 18.00 and 24.00 hours respectively.

Distribution of 3349 in Blood

Erythrocytes, leucocytes and plasma were separated from the pooled, citrated heart blood of 30 rats which had received 100 mgm. of 3349 base/kgm. from two to three hours earlier, and each was analysed in duplicate. The results from three such experiments, and from a similar one in a rabbit, are given in Table I.

TABLE I
Distribution of 3349 in the elements of the blood

Element	Rats A	Rats B	Rats C	Rabbit
Plasma ...	0.27	0.67	0.11	0.78
Whole blood ...	0.97	—	0.55	1.34
Red cells*	1.18	1.71	0.60	1.73
White cells*	2.90	39.1	3.65	149

* Concentrations in mgm./kgm. Otherwise in mgm./l

The chief features of these results may be briefly summarized as follows: 3349 is rather slowly absorbed in the rat, and attains only low concentrations in blood and plasma; the maxima from the oral administration of 100 mgm./kgm. are of the order of 0.8 and 0.2 mgm./l. respectively. The higher concentration in blood is associated with localization in red and white cells, although concentrations in the latter are variable. 3349 is rather persistent in the blood. The concentrations in tissues are about 100–300 times those in plasma; the highest after oral administration is found in the liver, the first main organ reached by the blood after leaving the capillaries of the villi. Analogously, the highest concentration immediately after intravenous administration is in the lung, although redistribution subsequently occurs to give a distribution rather similar to that following oral administration. Tissue concentrations from 25 mgm./kgm. intravenously and 100 mgm./kgm. orally are similar, suggesting that 3349 may be poorly absorbed when given by the latter route. 3349 persists strongly in the body, being still detectable in considerable amount six days after administration.

In nearly all these respects 3349 closely resembles mepacrine. Differences from the latter are difficult to assess by reference to the results of other workers; our own, which are as yet incomplete (Gage, Spinks and Tottey, to be published), suggest that mepacrine gives concentrations in blood and plasma rather similar to those of 3349, and in tissue about twice as high. It is considered that this difference may be associated with the greater length of the side-chain. The two drugs persist in about equal degree. Differences from quinine are partly illustrated by the plasma curve of the latter in diagram 1; quinine reaches much higher concentrations in blood, plasma and tissues, and is rapidly removed from the body.

Excretion of 3349 in the Rat

A. *Oral administration.* Six rats received 100 mgm. of 3349 base/kgm. by stomach-tube as a 2 per cent. solution of the dihydrochloride. They were kept for five days in a

metabolism cage, except for a feeding period of one hour daily, and faeces and urine were collected separately and analysed by the turbidimetric method. The results are given in diagram 4.

B. *Intravenous administration.* Six rats received 25 mgm. of 3349/kgm. intravenously, faeces and urine being collected separately over five days, under the same conditions as in the oral experiment, and analysed by the colorimetric method (diagram 4).

In both of these experiments only a small percentage of 3349 was excreted in the urine—a result in agreement with that already observed by us in man for 3349 (Spinks and Tottey, 1945*b*) and by other workers for mepacrine in man and animals (Tropp and Weise, 1933; Farinaud *et al.*, 1939; Dearborn *et al.*, 1943). Much larger amounts were excreted in the faeces, giving total recoveries of 63 per cent. and 43 per cent. in oral and intravenous experiments respectively. These are probably as high as could be expected if allowance is made for losses during the feeding periods and for drug still remaining in the tissues at the end of the experiment; it is therefore likely that little or none was metabolized.

An unexpectedly high percentage of the drug was excreted in the faeces following intravenous administration, and an attempt was made to obtain a more complete picture by analysing the intestinal contents of rats receiving the drug by this route. Groups of three rats, starved for 48 hours, received 25 mgm. of 3349 base/kgm. intravenously, and were killed at intervals after dosing. The small and large intestines, pooled separately, were slit longitudinally and the chyme was washed with distilled water into a beaker of water. The tissue was drained as thoroughly as possible, weighed, and homogenized in distilled water, the homogenate being analysed in the manner usual for tissue. The intestinal contents were homogenized on a ball mill and analysed as faeces. The results are given in Table II.

TABLE II
3349 in intestinal tissues and contents following the intravenous
administration of 25 mgm./kgm. in the rat

Time (hours)	Small intestine			Large intestine		
	Tissue		Contents	Tissue		Contents
	Mgm./kgm.	Total (mgm.)	Total (mgm.)	Mgm./kgm.	Total (mgm.)	Total (mgm.)
0.30	35.8	0.505	0.501	20.4	0.152	0.005
1.30	38.6	0.530	0.372	59.2	0.360	0.084
3.40	80.0	0.830	2.300	14.6	0.082	0.150
6.00	29.0	0.362	1.791	23.2	0.163	1.270
18.00	13.4	0.122	0.943	13.6	0.077	0.192
24.00	11.2	0.105	0.192	9.7	0.054	0.330

The efficiency of the washing procedure for the removal of the gut contents is uncertain and the degree of adsorption of 3349 on the gut wall is unknown, but the concentrations observed in intestinal tissue were of the same order as—probably lower than—those in other tissues examined. Assuming that removal of drug was complete, it follows that 3349 is excreted into the large intestine very slowly, and that it reaches the latter mainly by passage from the ileum. On the other hand, it is very rapidly excreted into the small intestine, either directly through the walls or in bile or other secretion. The excretion in

bile was examined separately: two rats were anaesthetized by intravenous injection of 100 mgm. of chloralose/kgm. and the bile ducts were cannulated so that the bile drained externally into a receiver; 20 mgm. of 3349 base/kgm. were then injected intravenously. Two control rats were anaesthetized and dosed in a similar manner without cannulation. One and a half hours after dosing both pairs of animals were sacrificed and blood, bile, tissues and intestinal contents were analysed, samples from the two animals of a pair being pooled. The results are given in Table III.

TABLE III
Concentrations of 3349 in bile and tissues of rats following the administration of 20 mgm./kgm. intravenously

Tissue	Cannulated rats	Control rats
Blood	1.70 mgm./l.	1.60 mgm./l.
Liver	79.2 mgm./kgm.	68.0 mgm./kgm.
Lung	124.0 "	128.0 "
Kidney	58.0 "	42.6 "
Spleen	35.2 "	35.4 "
Large intestine, wall	20.8 "	27.8 "
Small intestine, wall	49.4 "	39.4 "
Large intestine, contents	0.082 mgm.	0.091 mgm.
Small intestine, contents	0.235 "	0.770 "
Bile	0.273 "	—

Agreement between the two sets of rats was satisfactory, and concentrations in both were of the same order as those found in unanaesthetized animals. About half of the drug reaching the small intestine appears to do so by way of the bile; the remainder may be excreted through the walls of the intestine or in secretions other than bile.

Again the similarity to mepacrine and quinine is close, so far as the excretion of these in bile and faeces has been examined. Some workers on mepacrine (Tropp and Weise, 1933; Hecht, 1936) and quinine (Bernardbeig and Caujolle, 1935) have discussed the biliary excretion of these drugs as if it were a special property, and have stressed the fundamental nature of the enterohepatic cycle maintained by that excretion and subsequent reabsorption. However, sulphonamides (as well as many other drugs) are excreted in bile in relatively high concentrations (Spink *et al.*, 1941; Gough, 1943; Shay *et al.*, 1944) and must participate in an entirely analogous enterohepatic cycle; in view of their very different physico-chemical attributes and physiological disposition, it is to be doubted whether such a property can be regarded as characteristic of any particular chemical species. Nevertheless, the very large amounts of 3349 excreted in the faeces following intravenous administration suggest that the property may be particularly marked in the case of some basic antimalarials, being possibly associated with the high concentrations reached by these drugs in liver, pancreas and intestinal tissue.

Blood and Tissue Concentration of 3349 in Mice

The absorption and distribution of 3349 have also been examined in mice. The drug was administered to groups of six albino mice (20–30 gm.) in doses of 100 mgm. of base/kgm. by stomach-tube, and pooled blood and tissues were analysed at intervals after dosing. The results are given in diagram 5. Concentrations in blood were 1.76,

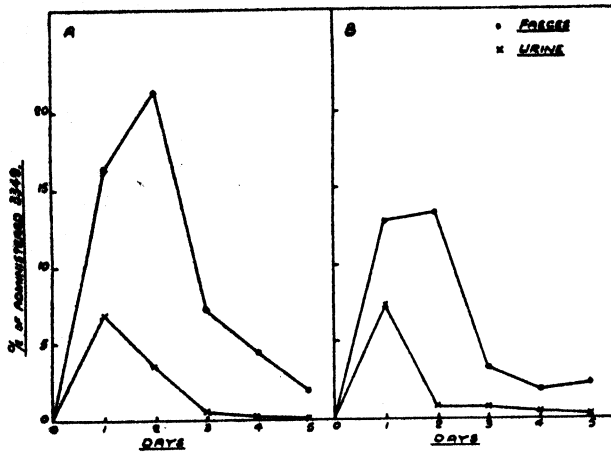


DIAGRAM 4. Excretion of 3349 in rat urine and faeces following the administration of 100 mgm. of base/kgm. orally (A) and 25 mgm. of base/kgm. intravenously (B).

DIAGRAM 5. Tissue concentrations of 3349 in the mouse following the oral administration of 100 mgm. of base/kgm.

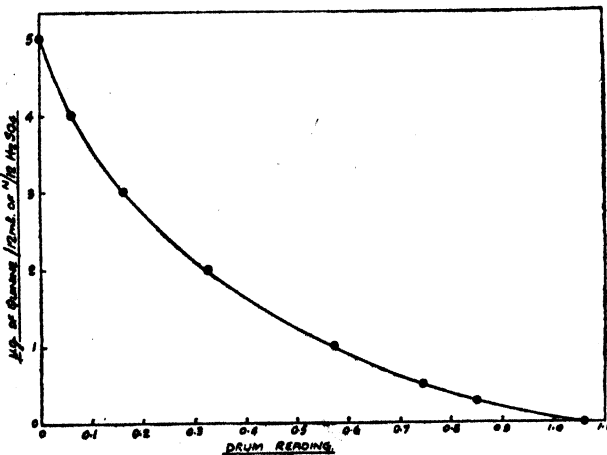
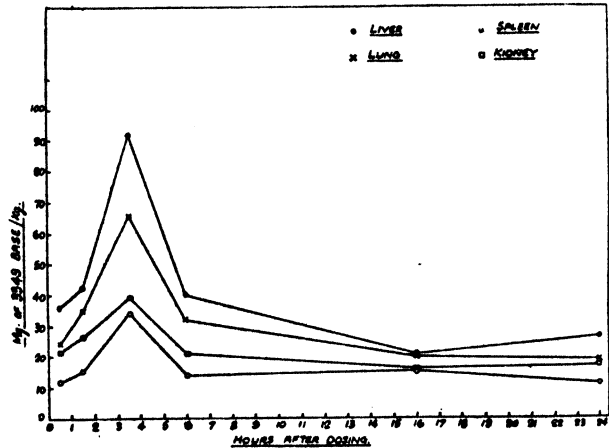


DIAGRAM 6. Standard curve for the determination of quinine, read on a modified Spekker fluorimeter having two photocells in parallel.

1.58, 2.58, 1.96, 1.50 and 1.33 mgm./l. after 0.30, 1.30, 3.30, 6.00, 16.00 and 24.00 hours respectively. In general, distribution and rates of absorption and clearance are similar in the two species, but blood concentrations in mice are two to three times those in the rat. Certain biguanides examined in these laboratories also reach higher concentrations in mouse than in rat blood (Spinks, to be published).

Determination of Quinine in Plasma

The following method for the determination of quinine in plasma was developed before that of Chen and Geiling (1944) had become available. It is not as convenient as the latter, and is not directly applicable to whole blood (from which recoveries are low), but it is much more sensitive (4-5 times), and may be of interest to workers who do not possess an electronic fluorimeter such as the Coleman.

Plasma (1 ml.) is treated with N/1 sodium hydroxide (1 ml.) and warmed at 50° for 20 minutes. After cooling, benzene (10 ml.) containing 2 per cent. of ethanol is added, and the mixture is shaken vigorously for five minutes and centrifuged for 15 minutes at 4,000 revolutions per minute. An aliquot of the solvent (8-9 ml.) is withdrawn and shaken with N/12 sulphuric acid (12 ml.) for three minutes. After centrifuging for five minutes at 2,000 revolutions per minute the lower layer is removed and read in the fluorimeter, using a blue filter, against a series of standards containing 0, 0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 μ gm. of quinine base/12 ml. of N/12 sulphuric acid (diagram 6). Recoveries of known amounts of quinine added to plasma are given in Table IV. Normal plasma gave no fluorescence.

TABLE IV
Recoveries of quinine from plasma (1 ml.)

Quinine added	Quinine found	Percentage recovery
μ gm.	μ gm.	
0.2	0.220	110
0.2	0.190	95
0.3	0.338	113
0.4	0.434	108
0.4	0.400	100
0.5	0.500	100
0.7	0.753	108
1.0	1.000	100
2.0	1.988	99
3.0	2.976	99
4.0	3.970	99
5.0	4.767	95
Mean recovery 102 per cent. \pm 3.7 ($p = 0.05$)		

DISCUSSION

The close similarity between 3349 and mepacrine in absorption, distribution and excretion is of great interest, particularly since they behave very differently in these respects from most other drugs which have been examined in similar detail. The differences are evidence of a selective affinity for cells, conditioning the localization of 3349 and mepacrine

in tissue at the expense of the plasma, and their slow excretion in the urine. It is reasonable to suppose that the same physico-chemical attribute which conditions these properties also plays a part in the antimalarial action of the drugs, permitting either their access to the parasite in adequate concentration or their participation in some enzyme process of that parasite. This physico-chemical attribute is undoubtedly associated with the basic side-chain, and may possibly take the form of a strong tendency towards adsorption at colloid surfaces (cf. Henry and Grindley, 1945). The present need is mainly for an investigation into the pharmacological properties of a series of basic antimalarials and the attempted relation of these properties to variation in some physico-chemical attribute such as pKb, degree of adsorption at colloid surfaces, lipid solubility, etc. Our own work will, it is hoped, follow these general lines.

SUMMARY

1. 3349 is absorbed slowly following oral administration, and reaches only low concentrations in blood and plasma; concentrations in glandular tissue, lung and intestinal tissue are 100–300 times those of plasma; it reaches the brain. Concentrations from intravenous administration are about the same as those from oral administration of four times the dose. The high blood/plasma concentration ratio is due to localization in red and white cells; concentrations in white cells are variable. 3349 persists strongly, being found in considerable amount in the tissues six days after a single intravenous dose.

2. 3349 is excreted mainly in the faeces, which it reaches partly by way of the bile. The total recoveries in urine and faeces indicate that it is probably not metabolized to a marked extent.

3. 3349 is distributed similarly in the mouse and the rat. Blood concentrations in the mouse are two to three times those in the rat.

4. 3349 closely resembles mepacrine in so far as they have been similarly studied.

5. A sensitive method of determining quinine in plasma is described.

ACKNOWLEDGEMENTS.—The authors wish to thank Dr. J. Raventos for assistance with biliary cannulations.

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STUDIES ON SYNTHETIC ANTIMALARIAL DRUGS

XVII.—THE ABSORPTION, DISTRIBUTION AND EXCRETION OF N₁-*p*-CHLOROPHENYL-N₅-METHYL-N₅-ISOPROPYLBIGUANIDE (4430) IN EXPERIMENTAL ANIMALS AND MAN

BY

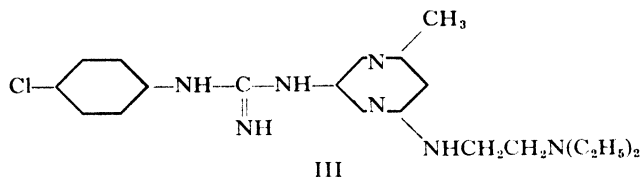
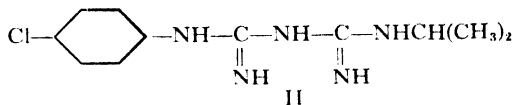
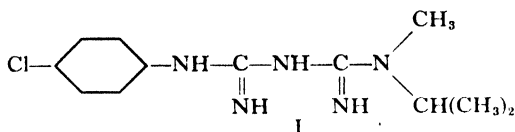
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(Received for publication February 18th, 1946)

INTRODUCTION

An earlier paper in this series (Curd, Davey and Rose, 1945; cf. Curd and Rose, 1946) described the development of the arylbiguanides, an entirely novel class of anti-malarial compounds showing high activity against both blood and exo-erythrocytic forms of several avian *Plasmodia*. The two most important members of this class are N₁-*p*-chlorophenyl-N₅-methyl-N₅-isopropylbiguanide (4430, I) and N₁-*p*-chlorophenyl-N₅-isopropylbiguanide (4888, Paludrine, II). The therapeutic action of 4430 in human malaria has been demonstrated by Adams *et al.* (1945).



From the pharmacological point of view the chief interest of 4430 and allied compounds lies in their simplicity of structure. The similar and characteristic physiological distribution of mepacrine and 3349 (III) (Spinks and Tottey, 1945*b*, 1946) is undoubtedly associated in large part with the presence in the molecules of dialkylaminoalkylamino side-chains, and possibly also with their polynuclear nature. Although the biguanides lack both these features, they also are strong bases, and it was consequently of great interest to examine their absorption, distribution and excretion under conditions permitting comparison with the behaviour of 3349 and mepacrine. This has now been done for 4430 in rat and mouse, and preliminary data are also presented on its absorption and excretion in rabbit and man.

EXPERIMENTAL SECTION

The absorption, distribution and excretion of 4430 were examined in rat and mouse under exactly the same conditions as those used for 3349 (Spinks and Tottey, 1946), except that the standard doses were reduced to 80 and 20 mgm. of base/kgm. orally and

intravenously respectively. This was necessary to facilitate comparison with some related biguanides that showed rather high toxicity. The drug was administered as a solution or dispersion of the acetate according to whether the desired strength was below or above the solubility of the latter in water (1.2 per cent.). It was determined throughout by the colorimetric method (Spinks and Tottey, 1945*a*, 1945*c*). In all diagrams and tables concentrations of 4430 are recorded as mgm. of base/l. or kgm.

A. *Blood, Plasma and Tissue Concentrations of 4430 Following Oral Administration in the Rat*

Groups of four albino rats (180–230 gm.) received 80 mgm. of 4430 base/kgm. by stomach-tube as a 2 per cent. dispersion of the acetate, and were sacrificed at intervals after dosing by withdrawal of heart blood under chloroform anaesthesia. Concentrations of 4430 found in pooled blood, plasma and tissues are recorded in Table I and diagram 1.

TABLE I
Blood, plasma and tissue concentrations of 4430 following the oral administration of 80 mgm. of base/kgm. in the rat

Time (hours)	Concentrations in mgm. of base/l. or kgm. in					
	Blood	Plasma	Liver	Lung	Spleen	Kidney
0.20	0.08	0.12				
0.30	1.52	0.18	9.10	3.45	2.60	3.20
1.00		0.44	10.52	4.01	1.80	6.08
1.30	0.77	0.46	27.30	10.12	7.55	12.42
2.30	0.53	0.23	26.50	4.70	5.50	4.70
3.30	0.52	0.28	13.58	2.96	4.05	6.28
5.00		0.11	9.48	7.64	6.34	10.62
7.00	0.38	0.12	4.74	4.48	—	3.85
16.00	0.22	0.13	5.92	8.15	2.98	3.00
30.00	0.15	0.07	3.07	3.56	1.81	3.00

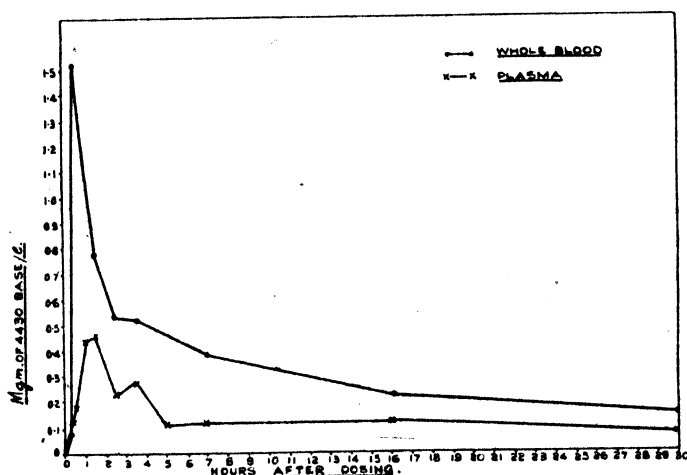


DIAGRAM 1. Blood and plasma concentrations of 4430 in the rat following the oral administration of 80 mgm. of base/kgm.

B. Blood, Plasma and Tissue Concentrations of 4430 Following Intravenous Administration in the Rat

Groups of four rats received 20 mgm. of 4430 base/kgm. intravenously as a 1 per cent. solution of the acetate, and were sacrificed at intervals. Concentrations found in pooled blood, plasma and tissues are given in Table II.

TABLE II
Blood, plasma and tissue concentrations of 4430 following the intravenous administration of 20 mgm. of base/kgm. in the rat

Time (hours)	Concentrations in mgm. of base/l. or kgm. in					
	Blood	Plasma	Liver	Lung	Spleen	Kidney
0-30	1.95	0.55	36.8	48.9	18.7	119.4
1-00	0.75	0.30	15.5	41.2	21.3	50.4
2-30	0.56	0.19	2.31	23.8	8.3	34.3
7-00	0.52	0.15	1.9	8.5	4.0	5.5
19-00	0.35	0.17	2.9	6.4	3.3	2.8

These results may be summarized as follows: 4430 is rapidly absorbed in the rat following the oral administration of 80 mgm. of base/kgm. and reaches maximum concentrations of about 1.5 and 0.45 mgm./l. in blood and plasma respectively. It is rather persistent at lower concentrations. The concentrations in tissue are much higher than those in plasma, the ratios being of the order of 10 to 50. Following intravenous administration, 4430 is rapidly distributed through the tissues, and initial concentrations in these are high, particularly in the kidney. The high concentration in kidney may be associated with the fact that 4430 is more readily excreted in the urine than mepacrine or quinine. 4430 is rather rapidly cleared from the tissues, the concentrations after 24 hours usually being less than 5 mgm./kgm. However, it is detectable in the urine at least five days after administration.

Compared with mepacrine and 3349, 4430 is more rapidly absorbed and more rapidly eliminated, and gives maximum concentrations about twice as high in blood and plasma. The most marked point of difference is the low tissue/plasma concentration ratio. Mepacrine, 3349 and quinine, when administered under exactly the same conditions, show tissue/plasma concentration ratios of 300-1,000, 100-300 and 50-200 respectively. Tissue in this sense includes those examined above; concentrations in muscle and fat may be much lower.

C. Excretion of 4430 in Rat Urine and Faeces

The excretion of 4430 in the urine and faeces of rats has been examined following the oral and intravenous administration of 80 and 20 mgm. of base/kgm. respectively to groups of six animals. These were kept in metabolism cages, except for feeding periods of one hour daily, and faeces and urine were collected separately in 24-hour samples. Amounts of drug found are recorded in diagram 2.

The percentage of 4430 excreted in the urine was low following both oral and intravenous administration, and, as with 3349, considerable amounts appeared in the faeces. Nevertheless, the ratios between faecal and urinary excretion differed considerably in

the two experiments, suggesting that much drug passes through the gut unabsorbed. The total recovery was of the order of 30 per cent. in each experiment—much lower than was achieved with 3349, using exactly the same technique. It is therefore possible that 4430 is largely metabolized by the rat.

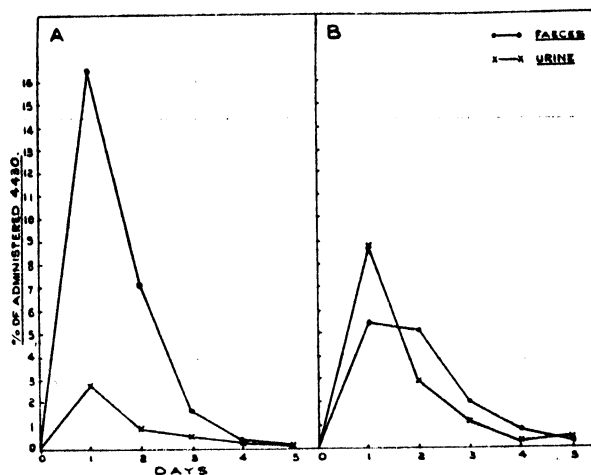


DIAGRAM 2. Excretion of 4430 in the urine and faeces of the rat following the oral administration of 80 mgm. of base/kgm. (A), and intravenous administration of 20 mgm. of base/kgm. (B).

The presence of 4430 in the faeces following intravenous administration showed that, like 3349, it must have been excreted from the blood into the intestine. The extent of such excretion was further examined by the methods already described for 3349 (Spinks and Tottey, 1946). Groups of four rats received 20 mgm. of base/kgm. intravenously, the separately pooled contents of large and small intestines being analysed at intervals after dosing. The results are given in Table III.

TABLE III
4430 in small and large intestines of rats following the intravenous administration of 20 mgm. of base/kgm.

Time (hours)	Small intestine		Large intestine	
	Mgm.	Percentage of dose	Mgm.	Percentage of dose
0.30	0.366	2.15	0.032	0.19
1.00	0.894	4.70	0.117	0.62
2.30	0.769	2.85	0.199	0.74
7.00	0.178	1.11	0.690	4.31
19.00	0.290	1.16	0.460	1.86

Here the similarity to 3349 is close. 4430 is excreted freely into the small intestine, but much more slowly into the large intestine, probably reaching the latter chiefly by passage from the ileum. Biliary cannulation showed that, as with 3349, excretion into the small intestine occurs partly by way of the bile. For this purpose, two rats (280, 300 gm.) were anaesthetized by intravenous injection of 100 mgm. of chloralose/kgm. and the

bile-ducts were cannulated so that the bile drained externally into receivers. 4430 was then injected intravenously in doses of 20 mgm. of base/kgm., and the bile was collected for one and a half hours. A total volume of 5.2 ml. was obtained from the two rats. They were then sacrificed, and pooled bile, blood, tissues and contents of small and large intestines were analysed, with the following results: blood, 0.687 mgm./l.; liver, 8.55 mgm./kgm.; lung, 19.3 mgm./kgm.; spleen, 8.54 mgm./kgm.; kidney, 39.1 mgm./kgm.; bile, 23.9 mgm./l. (0.125 mgm., 1.08 per cent. of dose); contents of small intestine, 0.212 mgm. (1.83 per cent. of dose); contents of large intestine, 0.497 mgm. (0.428 per cent. of dose). These concentrations agree well with those previously found in unanaesthetized animals (Tables II and III). Like 3349, 4430 reaches the small intestine partly by way of the bile and partly by excretion either through the walls of the intestine or in secretions other than bile. The non-specific nature of such excretion mechanisms has already been pointed out (Spinks and Tottey, 1946), but it was suggested that the antimalarials might differ quantitatively, if not qualitatively, from other drugs in the extent to which excretion into the intestine occurs. 4430 does not show this property to the same degree as 3349, as is indicated by the much smaller amounts excreted in the faeces (diagram 2) and by the lower drug content of small and large intestines following intravenous administration (Table III). This may possibly be due to the lower concentrations reached by 4430 in the tissues that are presumably associated with these excretion mechanisms, as, for example, liver, pancreas and intestine.

D. *Blood and Tissue Concentrations of 4430 in the Mouse*

4430 was administered by stomach-tube to groups of six albino mice (20-30 gm.) in doses of 80 mgm. of base/kgm. as a 0.4 per cent. solution of the acetate, and pooled heart blood and tissues were analysed at intervals after dosing (Table IV).

TABLE IV
Blood and tissue concentrations of 4430 in mice following the oral administration of 80 mgm. of base/kgm.

Time (hours)	Concentrations in mgm. of base/l. or kgm. in				
	Blood.	Liver	Lung	Spleen	Kidney
0.30	7.7	21.6	8.0	16.55	13.9
1.00	5.5				
1.30	2.8	60.5	15.1	10.4	16.7
2.30	1.75	103.0	26.75	26.75	37.0
4.00		20.5	17.8	6.0	12.3
7.00	1.3	29.0		6.1	6.0
16.00	1.2	16.9	19.3		
30.00		15.7	6.7	6.7	7.6
48.00	0.07	0.76	1.12	1.0	1.2

Like 3349, 4430 gives higher blood concentrations in mouse than in rat following the administration of the same dose. Distribution is rather similar in the two species, except that ratios between tissue concentration and blood concentration are somewhat higher in the mouse. In agreement with the results in the rat, 4430 is more rapidly absorbed and gives higher concentrations in the blood than 3349.

E. Absorption and Excretion of 4430 in the Rabbit

A typical absorption curve of 4430 in the rabbit is shown in diagram 3. A dose of 80 mgm. of base/kgm. was administered by stomach-tube as a 5 per cent. dispersion of the acetate. Concentrations in rabbit blood are between those in mouse and rat; absorption is slower in the rabbit than in the other two animals. The excretion of 4430 in rabbit urine and faeces following oral and intravenous administration is shown in Table V.

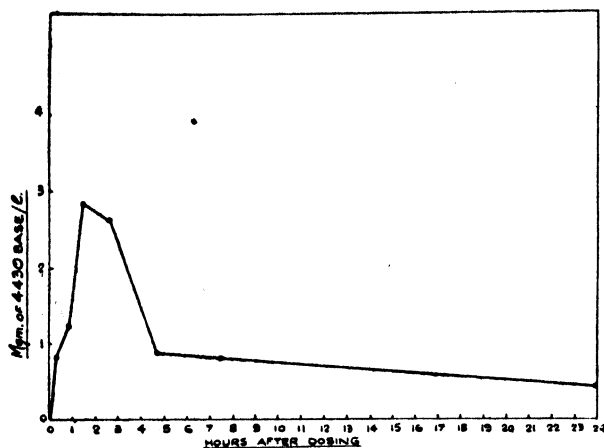


DIAGRAM 3. Blood concentrations of 4430 in the rabbit following the oral administration of 80 mgm. of base/kgm.

TABLE V

Excretion of 4430 in rabbit urine and faeces following the administration of 80 mgm. of base/kgm. orally and 8 mgm. of base/kgm. intravenously

Day	80 mgm./kgm. orally				8 mgm./kgm. intravenously			
	Urine		Faeces		Urine		Faeces	
	Mgm.	Percentage of dose	Mgm.	Percentage of dose	Mgm.	Percentage of dose	Mgm.	Percentage of dose
1	7.77	6.18	33.85	27.00	3.65	18.25	1.35	6.25
2	1.36	1.08	5.62	4.46	0.38	1.90	0.05	0.25
3	0.30	0.24	11.64	9.28	0.37	1.85	0.04	0.20
4	0.15	0.12	0.24	0.19	0.06	0.30	0.39	1.95
5	0.07	0.06	0.20	0.16	0.22	1.10	0.43	2.15
Total	9.65	7.68	51.55	41.09	4.68	23.40	2.26	10.80

The output of faeces was variable in both rabbits, and the excretion of drug therein was much more irregular than in the rat; but the general findings are very similar. Total excretion in urine and faeces was again low, about half and a third of the administered drug being recovered in oral and intravenous experiments respectively. As compared with 3349 in the same species (unpublished results), a greater percentage of drug is excreted in the urine and less in the faeces, in agreement with the general picture observed in the rat.

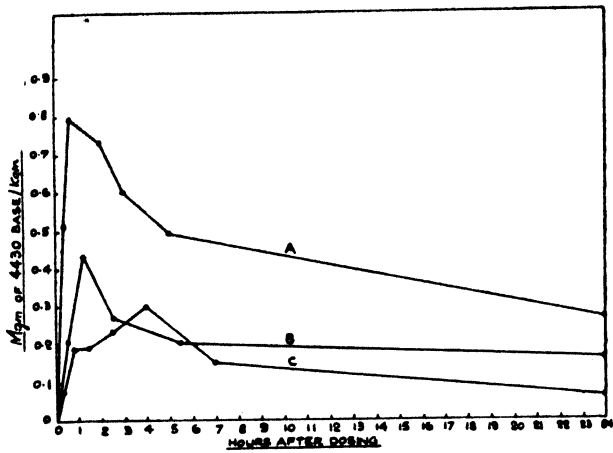


DIAGRAM 4. Blood concentrations of 4430 in human volunteers following the administration of single doses of 400 mgm. (A) and 200 mgm. (B, C) of the acetate.

DIAGRAM 5. Maximum blood concentrations of 4430 in human volunteers following the administration of 400 mgm. (D, J) and 200 mgm. (R, M) of acetate thrice daily.

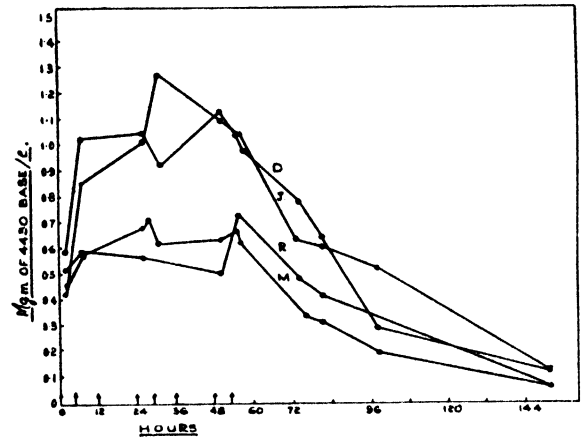
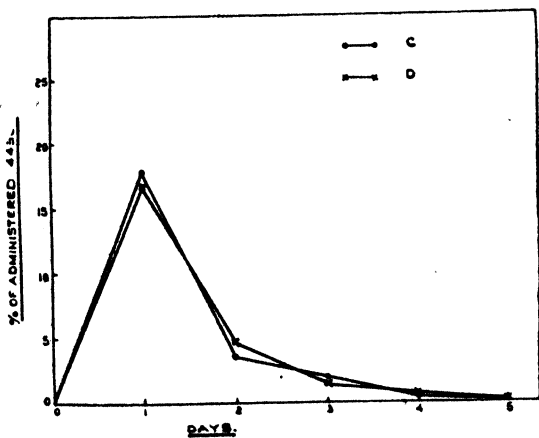


DIAGRAM 6. Excretion of 4430 in the urine of two human volunteers following the administration of single doses of 100 mgm. of acetate.



F. *Absorption and Excretion of 4430 in Man*

Blood concentrations reached by 4430 in man following the oral administration of single doses of 200 and 400 mgm. of the acetate are given in diagram 4. As in mouse and rat, the drug is rapidly absorbed and reaches concentrations much higher than those attained by mepacrine when given in similar amounts. The blood concentrations obtained on repeated administration were examined in four volunteers, two of whom received 200 mgm. and two 400 mgm. of acetate, thrice daily. Blood was withdrawn by venipuncture $1\frac{1}{2}$ – $2\frac{1}{2}$ hours after selected doses, this period being chosen as likely to give the maximum concentration. After eight doses the fall in concentration was examined at intervals up to 90 hours, when there was only about 0.12 mgm./l. in the blood of the two subjects who had received 400 mgm. t.d.s., and 0.06 mgm./l. in those who had received 200 mgm. t.d.s. Maximum build-up had been attained by the second or third dose in all subjects, and the maximum concentrations were about 1 mgm./l. from 400 mgm. t.d.s. and about 0.6 mgm./l. from 200 mgm. t.d.s. (diagram 5).

The excretion of 4430 in human urine was examined in two volunteers who received single doses of 100 mgm. of acetate. Twenty-four-hour urine was collected over five days and analysed, with the results shown in diagram 6. Total recoveries of drug were 24.5 and 24.2 per cent. in volunteers C and D respectively—much higher than would be obtained with mepacrine or 3349 (Spinks and Tottey, 1945b).

In each of these experiments the drug was administered as uncrushed 100 mgm. or 200 mgm. tablets of the acetate.

DISCUSSION

4430 shows, qualitatively at least, the characteristic pharmacological properties generally associated with a basic antimalarial. These may be summarized as follows:

1. Concentrations in whole blood and plasma are low.
2. Concentrations in whole blood are two to four times those in plasma.
3. Concentrations in glandular tissue and lung are much higher than those in plasma.
4. Excretion in the urine is slow, and the amount appearing therein is a small percentage only of the administered dose.
5. Considerable amounts are excreted from the blood into the intestine and removed from the body in the faeces.

The probability that this characteristic disposition in the host reflects a pronounced affinity of the drug for cellular structures, including its ability to reach the parasite in high concentration, has already been discussed (Spinks and Tottey, 1946), and is further confirmed by these results with 4430, and those more recently obtained with Paludrine (Spinks, to be reported). The alkylbiguanide side-chain may therefore be supposed to display the conductophoric function postulated by Magidson *et al.* (1934, 1936) for the dialkylaminoalkylamino side-chain of mepacrine and pamaquin. As a nitrogen-carbon system capable of displaying tautomeric and mesomeric effects (Curd, Davey and Rose, 1945; Curd and Rose, 1946), it also displays the parasitocidal function postulated by Magidson for the heterocyclic nuclei of mepacrine and pamaquin. The novel character of 4430 and Paludrine as antimalarials partly lies in the structural economy of this combination of functions in the alkylbiguanide chain. Nevertheless, 4430 differs quantitatively from mepacrine and 3349. Concentrations in blood and plasma are higher and those in

tissue lower. It is excreted more rapidly, and to a greater extent, in the urine; and smaller amounts appear in the faeces. Mepacrine, 3349 and quinine also differ from each other, and the four drugs can be arranged in the following series of decreasing affinity for cells: mepacrine, 3349, quinine, 4430. The reasons for these differences will, the author believes, be found to rest mainly on variation of the basic dissociation constant and of the length of the alkyl groups in the basic side-chain. Future work will be concerned mainly with an examination of the behaviour of a number of biguanides carrying different alkyl substituents in the N₅ position, in co-ordination with work on their physical properties.

SUMMARY

1. After the oral administration of 80 mgm. of base/kgm. in the rat, 4430 is rapidly absorbed but reaches maximum concentrations only of 1.5 and 0.45 mgm./l. in blood and plasma respectively. Concentrations in liver, lung, spleen and kidney are 10–50 times those in plasma.

2. After intravenous administration of 20 mgm. of base/kgm. in the rat, 4430 is rapidly distributed through the tissues; initial concentrations are high, particularly in lung and kidney.

3. 4430 is rather rapidly cleared from the body; concentrations in tissue after 24 hours are below 5 mgm./kgm. Nevertheless, it is detectable in the urine at least five days after administration.

4. 4430 is excreted in the urine to a greater extent than 3349 or mepacrine. About 25 per cent. of the administered drug was recovered in the urine of two human volunteers.

5. In the rat 4430 is excreted into the intestine, partly in the bile and partly by other routes. It is nevertheless excreted in the faeces to a lesser extent than 3349.

6. Distribution of 4430 in mouse and rat is similar, but blood concentrations in the mouse are higher than in the rat.

7. 400 mgm. of 4430 thrice daily in man give maximum concentrations in the blood of about 1 mgm./l., and 200 mgm. thrice daily about 0.6 mgm./l., both reached after the second dose.

8. Total recovery of 4430 in urine and faeces of rat and rabbit is less than 50 per cent.; it is probably metabolized by both species.

9. 4430 shows qualitatively similar distribution and excretion properties to quinine, mepacrine and 3349. The alkylbiguanide chain therefore displays a conductophoric function (cf. Magidson) similar to that of the dialkylaminoalkylamino side-chain of the latter drugs. It must also display the parasitidal function which in mepacrine, etc., is presumably associated with the heterocyclic nuclei.

10. 4430 differs quantitatively from mepacrine, 3349 and quinine in that it shows less affinity for cellular structures generally.

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SOME NOTES ON THE MAINTENANCE AND BREEDING OF SCHISTOSOME VECTORS IN GREAT BRITAIN, WITH SPECIAL REFERENCE TO *PLANORBIS* *GUADALOUPENSIS* SOWERBY

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In late 1945 plans were made under the auspices of the Medical Research Council to attempt the establishment in this country of a strain of schistosomiasis, with a view to initiating chemotherapeutic research on this infection.

As a preliminary, it was necessary to obtain and establish suitable species of molluscan intermediate hosts with which to work. Enquiry proved that the majority of such molluscan vectors as were formerly maintained in this country had died during the war, and that importation of snails from abroad would be necessary.

As a result of requests made to various quarters overseas, at least three vector species have now been maintained for several months in the Liverpool School of Tropical Medicine. The following paper deals with a few points connected with obtaining and maintaining the snails, and with their bionomics, with special reference to *Planorbis guadaloupensis*. As the establishment of a vigorous stock of breeding snails is a necessary first step in any programme of schistosomiasis research outside the endemic areas, it is felt that these notes may be of value to other workers.

SOURCE OF MATERIAL

Bullinus truncatus and *Planorbis boissyi* were obtained from Egypt through the kindness of Dr. Abdel Azim, of the Bilharzia Snail Destruction Section of the Egyptian Ministry of Public Health, and are discussed more fully later.

Through the kind co-operation of the War Office Department of Biological Research and the military authorities of the British Army on the Rhine, the writer was enabled to visit the laboratories of the I.G. Farben Werke in Elberfeld, Germany, where Kikuth and his co-workers have maintained *Schistosoma mansoni* in snails and mice since 1939. The snails were brought back to this country packed in wet sand, and, although the mortality was high, the survivors have bred freely and a strain appears to be now well established here.

THE NATURAL HISTORY OF *PLANORBIS GUADALOUPENSIS* SOWERBY

The old-established name of *Planorbis guadaloupensis* is here retained. In recent textbooks (Strong, 1942) the name is given as *Australorbis glabratus*, and certain authorities combine *P. guadaloupensis*, *P. olivaceus*, *P. centimetralis*, *P. antiguensis*, and possibly *P. pfeifferi*, together, under this newer name, as one species. It appears that, at best, *Australorbis* is no more than a subgenus of *Planorbis*. In this paper the old nomenclature and species differentiation are used.

Planorbis guadaloupensis is a large, olive-brown snail, flattened in the lateral plane

with five to five and a half whorls, and measuring 2.1×1.8 cm. in diameter when fully grown. The immature snails have a very brittle and light-coloured shell, with characteristic black spots on the upper half. The adults brought back from Germany were smaller than this (1.4×1.2 cm.), but grew larger when exposed to the warmth of a constant-temperature tropical room. This species bears a resemblance to the closely related *P. olivaceus*, *P. antiguensis* and *P. centimetralis*, and it has long been recognized as an important vector of *Schistosoma mansoni* in tropical America and the Antilles. Lutz (1918) records it as a vector in the Natal region and other parts of Brazil, and quotes Iturbe and Gonzalez as authorities for its rôle as the chief vector in Venezuela. He further records its occurrence in Puerto Rico, as well as in Guadaloupe. It has to be distinguished from the larger *P. olivaceus* and from the smaller *P. centimetralis*, both of which are important vectors, the latter especially around Pernambuco.

Germain and Neveu-Lemaire (1926) list *P. guadaloupensis* as the principal vector of *S. mansoni* in Venezuela, Puerto Rico and (quoting Brumpt) certain islands of the Antilles. They incriminate the closely related *P. antiguensis* as the vector in St. Kitts, St. Martin and Antigua. According to these authors, *P. guadaloupensis* also occurs in Haiti. In this connection it may be noted that Cameron (1928) has recorded the presence of *S. mansoni* in wild monkeys in St. Kitts.

Lampe (1927) has given a full account of the life-cycle of *S. mansoni* in Dutch Guiana, and he records that the highest infestation-rate observed in *P. guadaloupensis* was 15 per cent. (July, 1925), as compared with 18 per cent. in *P. olivaceus* (September, 1925). He considers that infection of the snail from swallowed ova rather than by direct penetration by miracidia is probable.

It would therefore appear that *P. guadaloupensis* is a widespread and important vector of schistosomiasis in tropical America and the Caribbean, sharing this rôle with *P. olivaceus*, *P. antiguensis* and *P. centimetralis*, which may be identical with it.

Of the specimens brought back from Elberfeld, all but six of the originals died, but a healthy stock has been raised from these. The snails are kept in an aerated aquarium with a mud bottom, in the tropical insect-room at a constant temperature of $23^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$ Under these conditions the breeding habits of the snails have been observed.

The snails were introduced to their new home on December 15th, 1945, and the first egg-nests appeared about December 30th. Most of the egg-nests are laid on the glass, less frequently on sprigs of *Elodea*. Attempts to remove egg-nests from the glass or on pieces of weed to a clean aquarium containing fresh boiled water were unsuccessful; the eggs died off after transfer, while those left alone in the main tank containing unboiled rain- or tap-water and weeds produced young. The first young emerged on or about January 21st, 1946, giving an incubation-period of about three weeks. By the middle of February, about six weeks after the first egg-nests were laid, developing young snails of all sizes were flourishing, and the snails could be said to be well established.

The egg-nest is an oval, colourless, translucent, gelatinous structure, without the fine circular striations of the egg-nests of some other species of snail, and contains a varying number of spherical, though often distorted, egg-sacs. The egg-sacs contain a pale yellow fluid, which gives the egg-nest a yellowish appearance. The number of egg-sacs present in one egg-nest is very variable, but an average-sized egg-nest measures 9×5 mm. and contains 30–45 egg-sacs. The smallest number counted in one egg-nest was 8 and the largest number was 105. The egg-sac has an average diameter of 0.89 mm. and

normally contains a single developing embryo, but occasionally two ova develop in the same egg-sac. The ovum measures 0.13 mm. in diameter. It commences segmentation on the first or second day, and produces a spherical, slightly motile embryo with a characteristic alveolar structure occupying most of the centre. By the third or fourth day the structure of the young veliger larva (0.45 mm. in diameter), showing a head with tentacles, and foot, shell and alveolar spiral, can be made out (fig. 2, A-H).

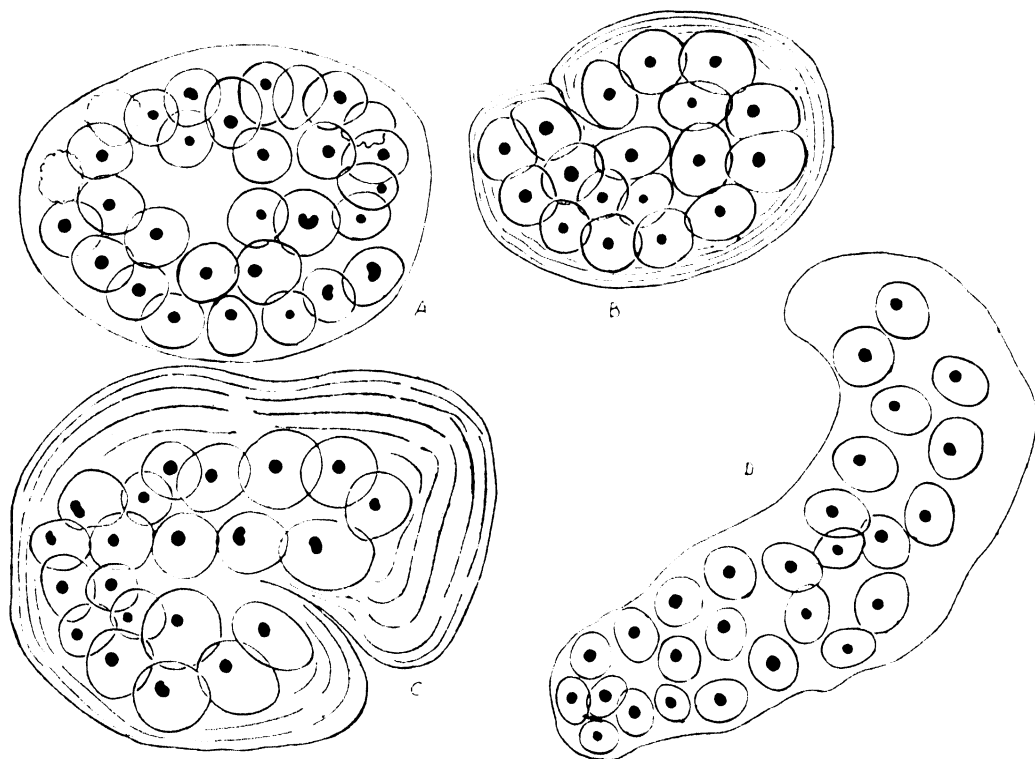


FIG. 1. A.—Egg-nest of *Planorbis guadaloupensis*; natural size 5 mm. \times 9 mm. B.—Egg-nest of *Bullinus truncatus*; natural size 5 mm. \times 6 mm. C.—Egg-nest of *Planorbis boissyi*; natural size 7 mm. \times 8 mm. D.—Egg-nest of *Lymnea* sp.; natural size 3 mm. \times 14 mm.

The fully developed veliger larva with eye-spots and the shell and soft-part structure of a young mollusc can be observed about the fourth or fifth day. At emergence the young snail has a diameter of 0.72 \times 0.71 mm. and shows the beginning of the spiral whorls of the shell.

For purposes of comparison, observations were made at the same time on the egg-nests and developmental stages of *Bullinus truncatus* and *Planorbis boissyi* from Egypt. The egg-nest of *Planorbis boissyi* is a little rounder than that of *P. guadaloupensis* and measures 7 \times 8 mm. It may have fine circular striations and is deeply notched; an average number of egg-sacs is 21. An egg-nest of *Bullinus truncatus* measures from 4 \times 3 mm., containing 7 egg-sacs, to 6 \times 5 mm., with 18 egg-sacs. It is notched, but less deeply

than the egg-nest of *P. boissyi*, and also has circular striations. Fig. 1 (A-D) shows the egg-nests of *P. guadaloupensis*, *P. boissyi* and *B. truncatus* for comparison with one another and with a figure of the characteristic elongated, curved, transparent, jelly-like egg-nest of *Lymnea* sp. The latter measures 14×3 mm., has no radial striations or notches, and contains 27 egg-sacs.

Blackie (1932), in considering the bionomics of the snail vectors of Southern Rhodesia, figures the egg-nests of *Physopsis globosa* and *Planorbis pfeifferi* and compares them with those of *Lymnea natalensis*. The nests of *Physopsis globosa* are very similar to those of *B. truncatus*, but are a little larger; the egg-nest of *Planorbis pfeifferi* is similar to that of *P. guadaloupensis*, but is a little smaller.

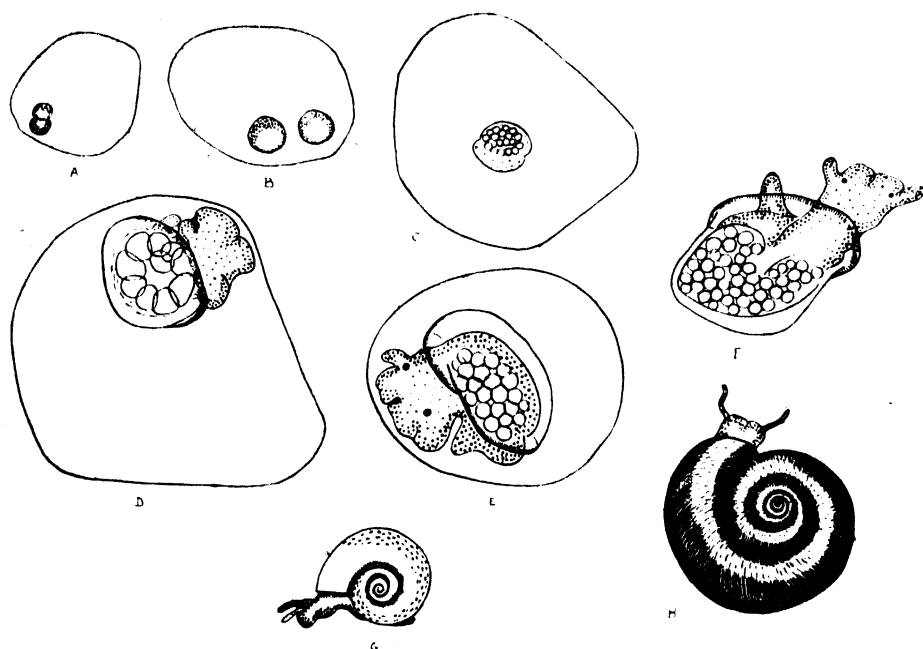


FIG. 2. Development of *Planorbis guadaloupensis*. A.—Segmenting ovum in egg-sac, 1st or 2nd day. B.—Two ova in same egg-sac. C.—Developing embryo. D.—Developing larva, 3rd-4th day. E.—Developing larva, 4th-5th day. F.—Young snail, just hatched. (For natural-size measurements, see text.) G.—*P. guadaloupensis* half-grown; natural diameters 0.5 cm. \times 0.4 cm. (note characteristic black spots). H.—*P. guadaloupensis* adult; natural diameters 2.1 cm. \times 1.8 cm.

For purposes of comparison, again, the developmental stages of *B. truncatus* were observed. Within two days of laying, the egg-sacs contained spherical embryos 0.21 mm. in diameter, with the characteristic alveolar centre. The developed embryos measured 0.42 mm. in their longest diameter. The egg-sacs themselves average 0.9–1.0 mm. in diameter. The newly hatched young snail (fig. 4, E) measures 0.8×0.8 mm.

By the third to fourth day, veliger larvae, sluggishly motile, with the commencement of a spiral whorl, can be observed; by the seventh to eighth day, well-formed veligers with eye-spots and a distinct shell can be seen; and after 10–11 days complete young snails can be made out, still within the egg-sacs (fig. 4, A–E).

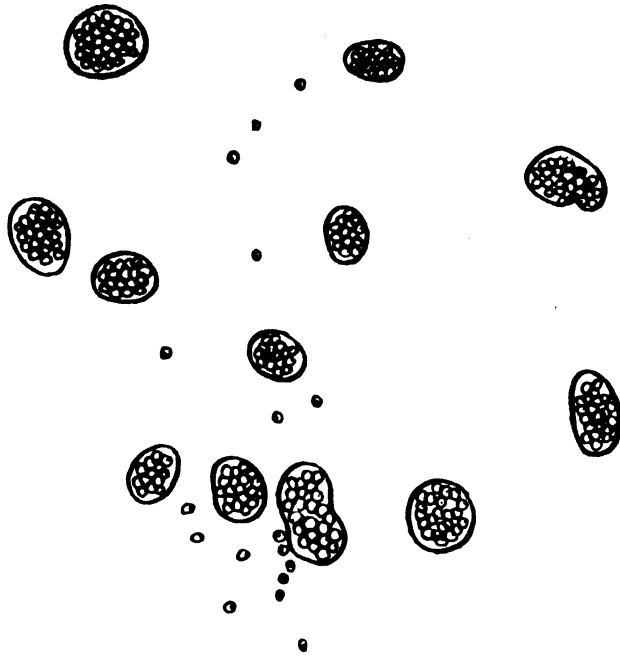


FIG. 3. Life-size tracing of egg-nests of *Planorbis guadaloupensis* on glass of aquarium, showing young snails escaping and spreading outwards from nest.

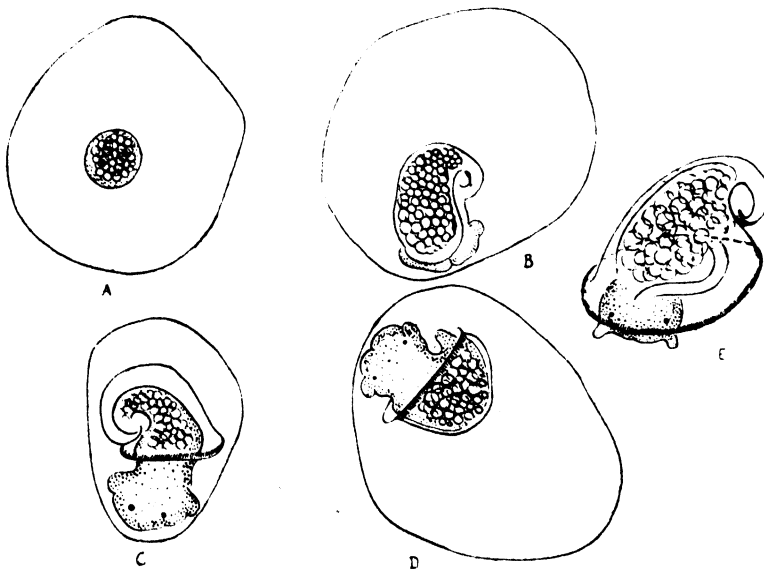


FIG. 4. Development of *Bullinus truncatus*. A.—Developing embryo within two days of laying. B.—Developing larva, 3rd-4th day. C.—Developing larva, 7th-8th day. D.—Developing larva, 10th-11th day. (For natural-size measurements, see text.) E.—Young snail, recently hatched; natural size 0.8 mm. \times 0.8 mm.

The following table summarizes the main points of difference in the egg-nests of *Planorbis guadaloupensis*, *P. boissyi*, *P. pfeifferi*, *Physopsis globosa*, *B. truncatus* and *Lymnea natalensis*.

TABLE

Species	Size, in mm.	Circular striations	Texture and appearance	Shape and form	Notch
<i>P. guadaloupensis</i>	9 × 5	—	Tough and translucent	Oval or irregularly circular	—
<i>P. boissyi</i>	8 × 7	+ or —	Tough and translucent	Irregularly kidney-shaped	+ Deep
<i>P. pfeifferi</i>	6 × 8	?	?Tough and translucent	Irregularly circular	—
<i>Ph. globosa</i>	8 × 5	?	Gelatinous	Kidney-shaped	+ Slight
<i>B. truncatus</i>	6 × 5	+	Tough and translucent	Oval, slightly kidney-shaped	+ Slight
<i>L. natalensis</i> } <i>Lymnea</i> sp. }	20 mm. or more long × 2.3 mm. wide	—	Clear and gelatinous	Elongated and curved	—

The specimens of *B. truncatus* and *P. boissyi* received from Egypt were placed in aquaria at a constant temperature of 23° C. \pm 1° C. on January 10th, 1946. The first egg-nests were laid by *B. truncatus* on January 17th and 18th, and by *P. boissyi* on January 19th to 21st. Fully formed moving veliger larvae of *Bullinus* were observed by January 28th, and complete young molluscs were present inside the egg-sacs by February 5th. The exact date of hatching was not observed, but young *Bullinus* were active in the aquarium by the second week in February, giving an incubation-period at this temperature of three to four weeks. The developmental stages of *P. pfeifferi* and *Ph. globosa* are fully figured by Gordon, Davey and Peaston (1934), and may be referred to for purposes of comparison.

TECHNIQUE OF MAINTAINING SNAILS

A few points are worth noting regarding the establishment of these snails for laboratory purposes.

1. *Aeration*. This is important, but it need not be absolutely continuous; nor is it necessary to employ a heavy air-pressure. In this case a small 'Dymax' electric motor pump was used, and, in order to avoid overheating, the machine was shut off for several hours daily. It is not necessary for the siphon-tubes to reach to the bottom of the aquarium, or indeed to pass more than a few inches below the surface. Many of the wild snails are naturally inhabitants of stagnant and foul pools.

2. *Feeding*. Lettuce-leaves were employed as the stock food; they are eaten with such avidity that a fresh supply was necessary at least every second day. No particular advantage was found in boiling young lettuce-leaves, though this has been recommended in the case of young snails. In this instance the snails seemed to show a preference for

unboiled leaves. Tough outside leaves should, however, be softened by light boiling. During the winter months lettuces are sometimes hard to obtain, and on these occasions 'mustard and cress' seedlings proved to be a satisfactory substitute. *P. guadaloupensis* is essentially a surface-feeding snail. At an early stage the young snails show a slow but steady tendency towards vertical migration upwards. Floating lettuce-leaves were therefore an adequate food-supply, as the snails concentrate on or just below the surface. *P. boissyi* is also in general a surface-feeder. *Bullinus truncatus* is more variable in its distribution in the aquarium and often remains on or near the bottom. For this reason it was found advisable to place one or two lettuce-leaves on the bottom of the aquarium, held down by a glass tube.

3. *Boiled versus unboiled water.* In certain localities it has been found that the tap-water contains a fungus (*Catenaria*) which attacks and destroys mollusc egg-nests, and for this reason boiled water has been recommended. In Liverpool, snails kept in rain- or tap-water, with weeds or mud, survived and bred best, while specimens of *B. truncatus* and *P. guadaloupensis* transferred to boiled water showed a higher mortality and less readiness to lay eggs. Nevertheless, some broods of egg-nests were entirely or almost entirely killed off by the fungus.

4. Certain snails, and especially *P. boissyi*, are inveterate wanderers. It is necessary, therefore, to keep aquaria containing *P. boissyi* covered with either glass or cardboard, and to shake down daily the snails which have climbed on to the 'roof' or to pick up those which have fallen over the side in an attempt to escape. These may be revived by placing them back in water and blowing water up the aperture with a pipette until all air is forced out of the shell and the snails sink.

5. In order to facilitate the examination of egg-nests without removing them from the sides of the aquarium it was found useful to place or suspend a number of glass microscope-slides inside the aquarium, as described by Gordon, Davey and Peaston (1934). *B. truncatus*, at least, would often lay its egg-nests on these slides, which could then be withdrawn and the ova examined under the microscope at various stages of development.

6. *Transporting live snails.* Snails should be packed in wet sand or mud, or tightly confined in wet blotting-paper. The sand or mud should not, however, be too saturated; the snails should only be lightly covered, and the box or tube should have ventilation apertures. In this way they will travel successfully for several days—for example, by air. On unpacking they should be placed in water at a temperature between 21° and 25° C. and the air should be pipetted out of the shells.

It is considered that *Planorbis guadaloupensis* is particularly suitable for use in this country in work on schistosomiasis. It breeds freely and easily in an aquarium, can be fed on easily obtainable foodstuffs, such as lettuce or cress, which need not be boiled, and in general requires very little attention. The writer feels that it might be worth while to attempt the establishment of a large stock of these snails in some zoological or botanical gardens, from which any future worker on schistosomiasis could draw material.

SUMMARY

The establishment of certain molluscan vectors of schistosomiasis in this country is described. The natural history of *Planorbis guadaloupensis* is summarized, and reasons are given why it is considered a suitable mollusc for experimental work.

ACKNOWLEDGEMENTS.—I am indebted to Professor T. H. Davey, of the Liverpool School of Tropical Medicine, in whose department this work was done; and to Professor R. T. Leiper, F.R.S., of the London School of Hygiene and Tropical Medicine, for helpful advice.

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CORRIGENDUM

'Some notes on the maintenance and breeding of schistosome vectors in Great Britain, with special reference to Planorbis guadaloupensis Sowerby,' by S.G. Cowper, vol.40, no.2, July, 1946.

Page 163, fourth and fifth lines from the bottom of the page: delete the words 'and possibly P.pfeifferi.'

A FURTHER NOTE ON THE NOMENCLATURE AND IDENTITY OF THE FROG FILARIA *FOLEYELLA LEIPERI* (RAILLIET, 1916)

BY

S. G. COWPER

(From the Department of Entomology and Parasitology, Liverpool School of Tropical Medicine)

(Received for publication March 4th, 1946)

In a recently published account of a filaria from the North American leopard-frog (*Rana sphenoccephala*) the present writer discussed the nomenclature and systematic position of the parasite at some length, and concluded that it was identical with the worm described by Leiper (1908) as *Filaria bufonis*, and renamed by Railliet (1916). In this paper (Cowper, 1945) it was concluded that the correct nomenclature should be *Foleyella leiperi* (Railliet, 1916), and that the species is synonymous with *Filaria bufonis* Leiper, 1908, with *Filaria leiperi* (Railliet, 1916), and possibly also with *Foleyella americanus* Walton, 1929. This work was done in 1939 but publication was held up by the war until 1945.

Since publication the writer has received a communication from Professor Morgan, of the Department of Veterinary Science, University of Wisconsin, drawing attention to the description of *Foleyella dolichoptera* sp. nov. and *F. brachyoptera* sp. nov. published by Wehr and Causey (1939). Owing to circumstances beyond his control the writer had not previously been able to consult this paper, and a reconsideration of the identity and position of this species of *Foleyella* now seems to be desirable.

In the following table the morphological characteristics of *Foleyella leiperi*, *Filaria bufonis* as described by Leiper, and *F. dolichoptera* and *F. brachyoptera* as described by Wehr and Causey, are compared, and a comparison of the microfilariae is included.

From this table the following deductions are drawn. *Foleyella brachyoptera* Wehr and Causey, 1939, differs from *F. leiperi* (Railliet, 1916) in the following particulars. The male of *F. brachyoptera* has six or seven pairs of post-anal papillae as against four pairs post-anal in *F. leiperi*, and the lateral alae extend only down to the level of the anus while in *F. leiperi* they extend the whole length. The oesophagus is somewhat shorter in *F. brachyoptera*.

In the female the entire worm is only 0.6–0.7 cm. long in *F. brachyoptera*, as against 5.0–5.5 cm. in *F. leiperi*. The microfilaria of *F. brachyoptera* is uniform in thickness throughout its length, while that of *F. leiperi* has a very well-marked caudal portion comprising two-thirds of its entire length.

For these reasons it seems to the writer that *F. brachyoptera* is a distinct species from *F. leiperi*, though both parasitize *Rana sphenoccephala*.

Turning to the second new species described by Wehr and Causey, *F. dolichoptera*, the males seem to be fairly identical with *F. leiperi* in their morphological characteristics. The female, however, is again a considerably smaller worm, i.e., 0.5–0.6 cm. against 5.0–5.5 cm. Assuming that the females described by Wehr and Causey were, in fact, fully grown adults, the difference between *F. dolichoptera* and *F. leiperi* would seem to lie in the length of the female and in the proportional size of the oesophagus. The comparison between the morphological details of *F. leiperi* and of *Filaria bufonis* described originally by Leiper from the Sudan in 1908, as shown in the following table, gives no grounds for modifying the conclusion that they are, in fact, the same species.

In conclusion, it appears that the nomenclature and synonymy of the frog filarias studied in Liverpool in 1939 are as stated (Cowper, 1945), viz., the correct nomenclature is *Foleyella leiperi* (Railliet, 1916), identical with *Filaria bufonis* Leiper, 1908, and synonymous with

Filaria leiperi (Railliet, 1916), possibly with *Foleyella americana* Walton, 1929, and probably with *F. dolichoptera* Wehr and Causey, 1939. *F. brachyoptera* Wehr and Causey, 1939, is a distinct species, while the validity of *F. dolichoptera* depends upon the total length of the female, the relative length of the oesophagus, and the cephalic papillae.

TABLE

	<i>Foleyella leiperi</i> (Railliet, 1916)	<i>Filaria bufonis</i> Leiper, 1908	<i>Foleyella brachyoptera</i> Wehr and Causey, 1939	<i>Foleyella dolichoptera</i> Wehr and Causey, 1939
MALE				
Length of entire worm	15-17 mm.	18 mm.	15-18 mm.	13-17 mm.
Length of oesophagus	1.7 mm.	Not stated	0.93-1.16 mm.	1.05-1.2 mm.
Length of long spicule	0.45-0.52 mm.	0.26 mm.	0.42-0.46 mm.	0.43-0.51 mm.
Length of short spicule	0.12 mm.	0.11 mm.	0.17-0.18 mm.	0.17-0.22 mm.
Lateral alae	Entire length	Not stated	Only to level of anal opening	Entire length
Anal papillae	6 pairs; 4 post-anal	6 pairs; 4 post-anal	6 or 7 pairs post-anal	4 pairs post-anal; rarely 5 on one side
FEMALE				
Length of entire worm	5.0-5.5 cm.	5.0 cm.	0.6-0.7 cm.	0.5-0.63 cm.
Anterior part of oesophagus	0.29 mm.	0.3 mm.	0.3-0.32 cm.	0.24-0.25 mm.
Entire oesophagus	1.72 mm.	1.5 mm.	1.57-1.72 mm.	1.39-1.5 mm.
Mouth to vulva distance	1.2 mm.	1.2 mm.	1.05-1.38 mm.	2.45 mm.
Lateral alae	Present	Not stated	Present	Not stated
Mouth—both sexes	Minute buccal teeth and tactile papillae	'Tiny touch corpuscles as in <i>F. perstans</i> '	4 pairs cephalic papillae	4 pairs cephalic papillae
MICROFILARIA				
	Narrow caudal portion $\frac{2}{3}$ of entire length Total length: 0.16-0.17 mm.	Not described	Diameter uniform throughout Total length: 0.13 mm.	Narrow caudal portion about $\frac{1}{2}$ total length Total length: 0.24 mm.

Further, Kreis (? 1945) has recently described two species of *Foleyella* from the Madagascar chameleon, viz., *F. chamaeleonis* and *F. pigmentata*. These may be added to the list given by Cowper (1945) together with *F. brachyoptera* and *F. dolichoptera*, thus bringing the number of described species of *Foleyella* up to 11.

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MEPACRINE IN ANIMAL TISSUES

BY

THE ARMY MALARIA RESEARCH UNIT,* OXFORD

(Received for publication March 11th, 1946)

In another paper (Army Malaria Research Unit, 1946) the distribution of mepacrine in blood is described. The experiments reported here form part of a further general study of the pharmacology of mepacrine undertaken by the Army Malaria Research Unit and previously reported to the Medical Research Council Malaria Committee. The experiments are complementary to those of Dearborn *et al.* (1943), Shannon *et al.* (1944) and the pioneer work of Hecht (1936).

Rats and dogs were given the drug, either by mouth or parenterally, and the mepacrine concentration in the various tissues and body fluids was determined. Observations were also made on the body fluids and hair of human volunteers receiving the drug by mouth.

METHODS

Mepacrine was estimated in tissues by the single-extraction procedure of Brodie and Udenfriend (1943) and in body fluids by the double-extraction method of Masen (1943) (Army Malaria Research Unit, 1945).

In the animal experiments the relevant tissue was dissected out and 50 mgm. weighed on a torsion balance. The tissue was then transferred to a mortar, ground with a small quantity of acid-washed sand and made alkaline, and from the resulting suspension the mepacrine was extracted with ethylene dichloride.

A. ANIMAL EXPERIMENTS

1. *Distribution of Mepacrine in Normal Animals Which Have Received the Drug by Mouth*

Table I shows the distribution of mepacrine in the tissues of three dogs,† and Table II the distribution in the tissues of three rats, after oral dosage of the drug. It is interesting to note that the tissues which take up the most mepacrine are those stained most readily with basic dyes. With the exception of the suprarenal, kidney and pancreas, these tissues are known to be rich in reticulo-endothelial elements.

2. *Mepacrine in Dog Tissues after Intravenous Injection of the Drug*

The concentration of mepacrine in the tissues of dogs after an intravenous injection of the drug is shown in Table III. All dogs had previously received the drug by mouth. Dogs 1, 2, 3 and 6 (first column) did not receive an injection, whereas the remainder were given an intravenous injection of the order of 10 mgm./kgm. Dogs 4, 5 and 9 (second column) were killed or died within 10 minutes of the injection. Dogs 8 and 10 (third column) were killed after 24 hours, and dogs 7 and 11 (fourth column) after 48 hours. It

* Professor B. G. Maegraith, Major G. M. Brown, R.C.A.M.C., Major R. J. Rossiter, R.A.M.C., Major K. N. Irvine, R.A.M.C., Capt. J. C. Lees, R.A.M.C., Capt. D. S. Parsons, R.A.M.C., Capt. C. N. Partington, R.A.M.C., Capt. J. L. Rennie, R.A.M.C., and Surgeon Lt. R. E. Havard, R.N.V.R.

† The dogs were given mepacrine as part of another experiment conducted by Professor J. H. Burn, F.R.S., of the Department of Pharmacology, Oxford.

TABLE I
Distribution of mepacrine in the tissues of dogs receiving the drug orally

	Dog 1	Dog 2	Dog 3
Weight of dog (kmg.)	7.8	7.7	4.7
Total previous dosage of mepacrine (gm.)	1.9 (by mouth)	2.5 (by mouth)	2.5 (by mouth)
Time of last dose before death (days)	2	5	6
Tissues :			
Liver ($\mu\text{gm.}/\text{gm.}$)	92	52	23
Spleen "	46	54	41
Lung "	41	60	12
Kidney "	25	23	13
Heart "	10	16	13
Brain "	4	4	7
Muscle "	3	11	17
Pancreas "	—	79	46
Suprarenal "	27	29	17

TABLE II
Distribution of mepacrine in the tissues of rats receiving the drug orally
(tissues examined 24 hours after last dose)

	Rat 1	Rat 2	Rat 3
Total previous dosage of mepacrine	50 mgm./kgm. for 5 days	50 mgm./kgm. for 8 days	50 mgm./kgm. for 8 days
Liver ($\mu\text{gm.}/\text{gm.}$)	1,740	2,230	1,680
Spleen "	508	721	699
Lung "	840	625	853
Kidney "	378	447	467
Heart "	189	142	278
Brain "	6	2	9
Muscle "	23	44	93
Pancreas "	244	123	259
Blood ($\mu\text{gm.}/\text{l.}$)	770	1,700	3,900

is seen that there was an enormous rise in the mepacrine concentration in the blood and plasma 10 minutes after the injection, but that by the end of 24 hours these high values had returned to normal. Ten minutes after the injection there was also a large rise in the concentration of the drug in the lung and kidney and, to a lesser extent, in the heart and spleen. The concentration in these tissues had fallen to normal again by the end of 24 hours. There was little change in the concentration of the drug in the liver.

The large increase in the mepacrine content of the lung and kidney indicates a true concentration of the drug in the tissue, since the increase in blood-level, although very great, is not sufficient in itself to explain this increase.

3. Mepacrine in Foetal Tissues

Dog 6 (see Table III) was pregnant, and Table IV gives the results of estimations done on similar organs from both the mother and the foetus. In the two organs studied the concentration of the drug in the maternal tissue was greater than that in the foetal. The experiment shows, however, that mepacrine is able to pass across the placental membrane.

TABLE III

Mepacrine content of organs of dogs after large intravenous injection of the drug

	Dog 1	Dog 2	Dog 3	Dog 6	Dog 4*	Dog 5	Dog 9	Dog 8	Dog 10	Dog 7	Dog 11
Weight (kgm.)	7.8	7.7	4.7	11.5	8.7	7.0	8.5	7.5	10.8	10.2	9.0
Total previous dosage of mepacrine by mouth (gm.)	1.9	2.5	2.5	4.0	2.9	6.4	0	6.6	3.2	5.0	0
Mepacrine given intra- travenously (mgm.)	0	0	0	0	180	180	120	120	78	120	108
	No intravenous injection				10 minutes after intra- venous injection			24 hours after intravenous injection		48 hours after intravenous injection	
Liver (μ gm./gm.)	92	52	23	45	37	64	45	139	67	37	46
Spleen "	46	54	41	—	131	—	—	218	94	30	53
Lung "	41	60	12	41	270	559	640	54	40	52	51
Kidney "	25	22	13	—	147	540	270	76	64	82	22
Heart "	10	16	13	—	118	159	134	21	35	16	6
Blood (μ gm./l.)	—	—	268	515	120,000	84,000	40,000	416	625	162	560
Plasma "	—	—	55	—	70,000	21,000	11,000	164	—	81	—

* The concentration of the drug in other organs of dog 4 was (μ gm./gm.): brain, 7; muscle, 15; pancreas, 139; suprarenal, 78.

TABLE IV

Concentration of mepacrine in maternal and foetal organs of dog ;
total dose before death, 4 gm.

Organ	Maternal	Foetal
Tissue :		
Lung (μ gm./gm.)	41	12
Liver "	45	27

4. Mepacrine in Bile

Table V gives the mepacrine content of gall-bladder bile removed after death from four dogs which had been receiving mepacrine by mouth for varying periods of time. It is seen that the mepacrine concentration in this bile was high; but it does not necessarily follow that the mepacrine secreted in the bile is lost to the body. It is possible that a considerable quantity of this mepacrine is reabsorbed during its passage through the gastro-intestinal tract.

5. Concentration in Tissues after Oral Dosage in the Rat

Nine rats were divided into three groups of three animals, and each was given a single dose of 15 mgm./kgm. mepacrine. One group was killed at the end of one hour, one at the end of three hours and one at the end of six hours. Table VI shows that after one hour the drug was present in all the tissues studied, with the greatest concentration

TABLE V
Mepacrine content of gall-bladder bile from dogs receiving mepacrine

Dog	Previous dosage of mepacrine	Weight (kgm.)	Concentration of mepacrine in bile (mgm./l.)
A	1.9 gm. in 4 weeks	10.3	43.0
B	2.5 gm. in 5 weeks	7.7	26.0
C	2.5 gm. in 8 weeks	4.7	9.0
D	2.9 gm. in 6 weeks	8.7	4.4

of the drug in the liver. After three hours, and again after six hours, the concentration of the drug in each of the tissues was increased, but the relative concentration in these tissues remained unchanged.

TABLE VI
Mepacrine concentration in tissues of rats
(expressed in $\mu\text{gm./gm.}$) after receiving a 15 mgm./kgm. dose of the drug

Time after dose	Liver	Spleen	Lung	Kidney
1 hour	41	8	26	16
	29	5	17	12
	37	2	14	6
	Mean 36	5	19	11
	—	—	—	—
3 hours	48	11	34	31
	—	21	56	48
	150	58	58	34
	Mean 99	30	49	38
	—	—	—	—
6 hours	129	53	74	51
	210	56	96	56
	170	58	128	45
	Mean 170	56	99	51
	—	—	—	—

B. HUMAN EXPERIMENTS

1. *The Excretion of Mepacrine in Sweat*

In order to investigate the loss of mepacrine in the sweat, two experiments on human volunteers were carried out. In the first experiment (10 subjects) each volunteer was placed for two hours in a large radiant-heat cradle which had been modified for use as a sweating-box. The volunteer was wrapped in rubber sheets, and the sweat which ran off was collected through an opening at his feet. At the end of two hours he was washed down with about 2,000 c.cm. distilled water, and the washings were collected for mepacrine estimation. The total volume of sweat was calculated from the weight of the volunteer before and after the experiment.

In the second experiment (five subjects) sweat was collected from each volunteer

during the course of a four-hour period of work in a tropical chamber, and the mepacrine was estimated in this undiluted specimen. The urine excreted during the same period was also collected for mepacrine estimation.

The results of the first experiment are shown in Table VII and those of the second in Table VIII. The concentration of mepacrine in the sweat was lower when sweating was due to heat alone, but the difference was not statistically significant. In both cases the amounts excreted were very small. The mepacrine concentration in sweat was of the same order as that in whole blood, but only 1/5 to 1/20 of that in urine. There is little correlation between the concentration in plasma and that in sweat ($P = 0.4$). An important practical conclusion is that the mepacrine concentration in the sweat is so low that the amount of mepacrine lost to the body by this route is negligible.

TABLE VII
Excretion of mepacrine during sweating induced by radiant heat

Volunteer no.	No. of days on mepacrine 0.1 gm. daily	Mepacrine concentration in			Calculated volume of sweat (c.cm.)	Mepacrine excreted in sweat in 2 hours (μ gm.)
		Blood (μ gm./l.)	Plasma (μ gm./l.)	Sweat (μ gm./l.)		
242	54	87	28	96	1,170	112
252	96	120	16	71	1,120	80
265	12	29	0	17	777	13
268	21	100	7	41	930	38
270	21	103	10	34	850	29
272	13	17	6	33	975	32
273	16	—	12	36	725	26
296	41	—	31	52	1,560	81
298*	36	—	—	44	870	38
305	24	94	9	79	1,190	94

* This volunteer spent only 1½ hours in the sweating-box.

TABLE VIII
Excretion of mepacrine during work in the tropical chamber

Volunteer no.	No. of days on mepacrine 0.1 gm. daily	Mepacrine concentration in		Mepacrine concentration in collected sweat (μ gm./l.)	Calculated volume of sweat (c.cm.)	Mepacrine excreted in 4 hours	
		Blood (μ gm./l.)	Plasma (μ gm./l.)			Sweat (μ gm.)	Urine (μ gm.)
244	42	128	66	410	1,720	705	3,600
246	40	122	55	90	1,690	152	3,100
268	28	142	22	148	840	123	1,140
270	27	145	24	53	1,120	59	550
286	41	176	33	170	1,020	173	1,440

2. The Excretion of Mepacrine in Saliva

Mepacrine is excreted in small quantities in the saliva, and this may account for the persistent taste of the drug which is sometimes reported. The concentration in the saliva is usually greater than that in the plasma and may be greater than that in whole blood. The salivary concentration seems to bear no constant relation either to that in

the plasma or to that in whole blood. Typical concentrations of mepacrine in the saliva of volunteers who had been receiving 0.1 gm. mepacrine daily for a number of months are given in Table IX.

TABLE IX
Mepacrine content of saliva of volunteers receiving mepacrine,
compared with that of blood and plasma

Mepacrine concentration ($\mu\text{gm./l.}$)		
Saliva	Blood	Plasma
120	290	25
165	120	60
70	120	20
30	140	50
100	110	15
190	190	60
60	—	65
80	—	34
270	—	26
12	—	25
96	—	18

3. The Excretion of Mepacrine in Seminal Fluid

Semen was obtained from two volunteers during a full course of mepacrine therapy (4.6 gm. in 12 days), and from one of these volunteers a further specimen was obtained a week after he had stopped taking the drug. Table X gives the concentration of mepacrine in seminal fluid compared with that of other body fluids. The concentration is higher than that of either whole blood or plasma, but is nothing like as high as that of the urine. The table suggests that the concentration of mepacrine in the seminal fluid is high if the concentration in the other body fluids is high. Since spermatozoa take up basic dyes, and since mepacrine generally behaves as such in the body, this result is hardly surprising.

TABLE X
Mepacrine content of seminal fluid of volunteers receiving
mepacrine, compared with that of other body fluids

Volunteer	Concentration of mepacrine ($\mu\text{gm./l.}$)			
	Seminal fluid	Blood	Plasma	Urine
A	1,120	340	62	14,900
B ₁	480	250	75	3,300
B ₂	420	110	24	3,300

4. Mepacrine Concentration in Human Bile

Two patients with post-operative biliary fistulas were given 0.1 gm. mepacrine hydrochloride orally daily for three days. The mepacrine concentrations in whole blood, plasma and 24-hour specimens of bile and urine were determined in both cases.

The mepacrine concentration in bile samples was also measured in each patient 1, 2, 4 and 8 hours after taking the first tablet. The detailed results are set out in Table XI.

Mepacrine appeared rapidly in the bile, the maximum concentration being reached 2-4 hours after the patient received the tablet. The concentration of mepacrine in the bile in both patients was higher than that in either whole blood or plasma, but was much less than that in urine.

Since the volumes of urine and bile eliminated in 24 hours were comparable, it follows that the total amount of mepacrine excreted in the urine was much greater than that lost in the bile. We had no means of ascertaining how much of the drug eliminated in the bile was reabsorbed from the gastro-intestinal tract.

TABLE XI
Mepacrine concentration in blood, plasma, bile and urine

	Concentration ($\mu\text{gm./l.}$)				Volume in 24 hours (c.cm.)		Total mepacrine eliminated (mgm.)	
	Blood	Plasma	Bile	Urine	Bile	Urine	Bile	Urine
Patient 1								
Day 1*	45	35	110	388	520	388	57	250
Day 2*	60	45	275	368	537	368	147	660
Day 3*	75	30	300	265	495	265	149	635
Day 4	125	80	380	412	340	412	126	865
Day 5	—	—	320	795	28	795	9	520
Patient 2								
Day 1*	80	40	180	800	385	620	69	496
Day 2*	—	80	260	950	525	715	136	680
Day 3*	—	—	280	2,000	580	350	162	700
Day 4	100	35	240	650	542	1,035	125	670
Day 5	—	—	250	950	550	570	138	540
Day 6	90	50	260	1,000	550	520	143	520

* 0.9 gm. mepacrine hydrochloride given orally 24 hours before estimation.

5. Mepacrine Concentration in Human Hair

Table XII shows the mepacrine concentration in the scalp hair of seven female volunteers who had received 0.1 gm. mepacrine per day for a period of six months. A small lock of hair 6-9 inches long was removed, and estimations were done on the proximal and distal $1\frac{1}{2}$ inches. It is seen that the concentration in the proximal portion of the hair, grown while the volunteer was receiving the drug, was much greater than that of the distal portion, probably grown before the experiment had begun. It will be seen that dark hair contained significantly higher concentrations of the drug than fair hair.

6. Mepacrine Concentration in Human Spleen

A patient* suffering from thrombocytopenic purpura was given 0.1 gm. mepacrine per day for 14 days. On the 15th day her spleen was removed and the concentration of mepacrine in the organ was found to be 47 $\mu\text{gm./gm.}$

* The patient was under the care of Professor L. J. Witts.

TABLE XII

Mepacrine concentration ($\mu\text{gm./gm.}$) in human hair

Volunteer	Proximal portion	Distal portion —
Brunette 1	85	0.5
" 2	51	0.5
" 3	54	3.5
" 4	67	1.0
" 5	29	3.0
Blonde 6	11	0
" 7	12	0.5

SUMMARY

1. The distribution of mepacrine in the tissues of dogs and rats that have received the drug by mouth has been investigated. The greatest drug concentration is in the liver, spleen, lung and suprarenal gland, with lower concentrations in the kidneys and pancreas; the lowest drug concentration was in the brain, muscle and heart.

2. After dogs had been given a single intravenous injection of mepacrine there was an immediate rise in the concentration of the drug in most tissues. This had fallen to normal by the end of 24 hours.

3. Mepacrine is able to pass the placental membrane. The concentration of the drug in both foetal and maternal organs from an animal that had received the drug by mouth was determined.

4. The mepacrine concentration in the bile of four dogs which had received various doses of mepacrine by mouth was 4.4–43.0 $\mu\text{gm./litre}$.

5. Mepacrine was detected in the organs of rats one hour after receiving a dose of 15 mgm./kgm. by mouth.

6. The mepacrine concentration in human sweat varied from 17 to 410 $\mu\text{gm./litre}$. It is concluded that only negligible amounts of mepacrine are excreted through the skin.

7. The mepacrine concentration in the saliva of volunteers taking 0.1 gm. daily was found to be of the same order as that in whole blood.

8. The mepacrine concentration in human semen was found to be higher than that of whole blood but much lower than that of urine.

9. The mepacrine concentration and output in bile was measured in two patients with biliary fistula. The total amount eliminated in bile represented only a small fraction of that excreted in the urine.

10. Mepacrine is excreted in the human hair. The concentration of the drug in the hair from subjects taking 0.1 gm. daily was determined. A greater concentration of the drug was found in dark hair than in fair hair.

11. The concentration of mepacrine in the spleen from a patient suffering from thrombocytopenic purpura, who had received 0.1 gm. mepacrine daily for 14 days, was 47 $\mu\text{gm./gm.}$

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DISTRIBUTION OF MEPACRINE IN BLOOD

BY

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Mepacrine estimations have been made by various authors on whole blood, serum and plasma. The content of mepacrine in each of these fluids on any given dosage régime differs considerably, and there seems to be no general agreement as to which gives the best estimate of the chances which the subject under investigation has of suppressing malaria. Shannon *et al.* (1944) demonstrated that on a given dosage the concentration of mepacrine in the white cells is very much greater than that in either the red cells or the plasma—an observation fully confirmed by us. For this and other reasons these workers decided to measure the mepacrine concentration in plasma rather than in whole blood. On theoretical grounds the argument seemed incontestable, but, before embarking upon a programme of work involving the measurement of the concentration of mepacrine in plasma only, it was decided to reinvestigate the matter.

We have found that the concentration of mepacrine in the white-cell layer of centrifuged blood is of a different order from that in either the red cells or plasma. There is a highly significant correlation between the mepacrine concentration in plasma and that in the red cells, whereas the correlation between the mepacrine concentration in the whole blood and that in the red cells is insignificant. This means that the concentration of mepacrine in the plasma gives a much better estimate of the mepacrine-level in the red cells than does the concentration in whole blood. In addition, it has been shown that the concentration of mepacrine in whole blood is more closely correlated with the concentration of the drug in the white-cell layer than with the concentration in the red-cell layer. It follows, therefore, that, if it is desired to obtain an estimate of the mepacrine concentration in the red cells, plasma determinations are of more value than determinations on whole blood.

It has also been possible to approach the problem in another way. Patients suffering from leukaemia, with abnormally high white-cell counts, have been given mepacrine, and the whole-blood and plasma mepacrine concentrations have been compared with those of normal persons receiving the same dosage of the drug. The whole-blood mepacrine concentrations of the patients with abnormally high white-cell counts was found to be of an altogether different order from those of normal persons, while the concentration of drug in the plasma was of the same order.

METHODS

Mepacrine was determined by a modification of the method of Masen (1943) (Army Malaria Research Unit, 1945). Potassium oxalate was used as an anticoagulant, since the ammonium ion of Wintrobe's salt mixture causes a liberation of mepacrine from the white cells into the plasma (Army Malaria Research Unit, 1945).

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DISTRIBUTION OF MEPACRINE IN NORMAL BLOOD

Freshly drawn blood was pipetted into a waisted centrifuge-tube, the volume being so adjusted that, after spinning, the white-cell layer occupied the upper part of the constriction in the tube. After spinning, the plasma was withdrawn and immediately recentrifuged. The tube was broken at the upper and lower limits of the white-cell layer and the mepacrine content of this layer was estimated. Estimations were also made on the packed red cells in the bottom of the tube, on the recentrifuged plasma, and on a further sample of the whole blood.

The results of eight such determinations, together with the two published observations of Shannon *et al.* (1944), are given in Table I. Each estimation was made on blood from a different subject, each subject being on a different mepacrine-dosage régime. This enabled observations to be made on bloods with widely different mepacrine content and, possibly, with a different mepacrine distribution. In some cases the haematocrit, haemoglobin and white- and red-cell counts were also determined. These data are given in Table II. Knowing the haematocrit reading and the mepacrine concentration in the plasma and

TABLE I
Mepacrine concentration (in $\mu\text{gm./l.}$) in different fractions of blood

No.	Previous mepacrine history	Mepacrine, in $\mu\text{gm./l.}$			
		Whole blood	Plasma	Red-cell layer	White-cell layer
*1	0.1 gm. mepacrine 3 times daily ...	291	90	149	9,500
*2	0.1 gm. mepacrine 3 times daily ...	551	89	117	18,400
3	0.1 gm. mepacrine daily for 8 weeks ...	174	18	41	5,600
4	0.1 gm. mepacrine daily for 8 weeks ...	132	17	35	8,300
5	0.1 gm. mepacrine daily for 4 weeks ...	108	31	86	—
6	0.1 gm. mepacrine daily for 4 weeks ...	208	40	60	9,600
7	0.1 gm. mepacrine 3 times daily for 7 days ...	336	57	114	14,300
8	0.1 gm. mepacrine 3 times daily for 7 days ...	232	100	193	7,900
9	1½–2 hours after first dose of 0.1 gm. mepacrine ...	37	2	6	5,400
10	1½–2 hours after first dose of 0.1 gm. mepacrine ...	43	11	13	1,900

* Figures of Shannon *et al.* (1944).

TABLE II
Additional data obtained from subjects referred to in Table I

No.	Haematocrit	White-cell count (cells/c.mm.)	Red-cell count (cells/c.mm.)	Haemoglobin
4	43	18,900	—	—
5	44	7,400	5,790,000	99
6	47	8,400	5,040,000	97
7	39	14,100	4,410,000	92
8	—	7,300	5,310,000	97
9	43	—	5,320,000	92
10	45	—	5,260,000	93

TABLE III
The distribution of mepacrine in whole blood

			Subject no.					
			4	5	6	7	9	10
<i>Given :</i>								
Haematocrit			43	44	47	39	43	45
Concentration of mepacrine			132	108	208	336	37	43
(in $\mu\text{gm./l.}$) in			17	31	40	57	2	11
	Whole blood		35	86	60	114	6	13
	Plasma							
	Red cells							
Content ($\mu\text{gm./l.}$) calculated			10	17	21	35	1	6
	Plasma		15	38	28	44	3	6
	Red cells							
Content ($\mu\text{gm./l.}$) of white-cell layer								
(i.e., whole-blood content less that								
of plasma and red cells)			107	54	159	257	33	31
<i>Percentage distribution :</i>								
Whole blood			100	100	100	100	100	100
Plasma			8	16	10	10	3	14
Red-cell layer			11	35	13	13	8	14
White-cell layer			81	49	77	77	89	72

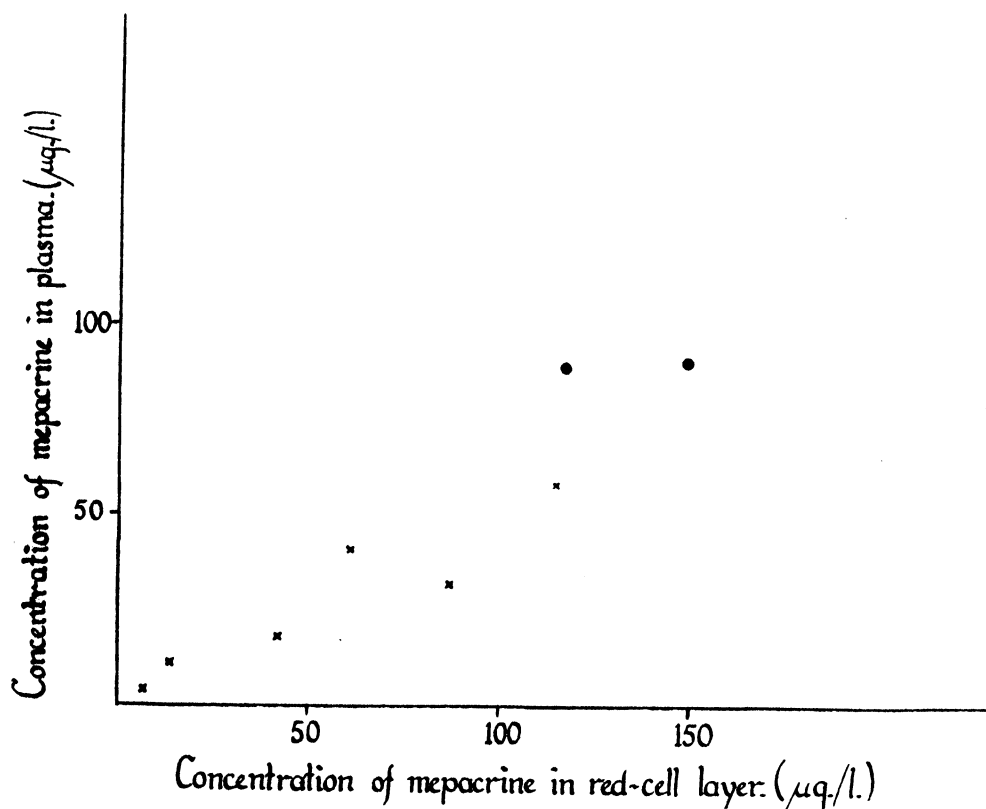


FIG. 1. Relation between concentration of mepacrine in plasma and in red-cell layer.
x = Observed values; ● = Values of Shannon *et al.* (1944).

red cells, it was possible to calculate the quantity of mepacrine in both the plasma and the red cells obtained from a litre of blood. The difference between this calculated value and the value derived from the observed whole-blood mepacrine concentration gives the mepacrine content of the white-cell layer. This calculation was performed in the seven cases for which the data were available (Table III), and it shows that, of the mepacrine in the whole blood, approximately 80 per cent. may be in the white-cell layer.

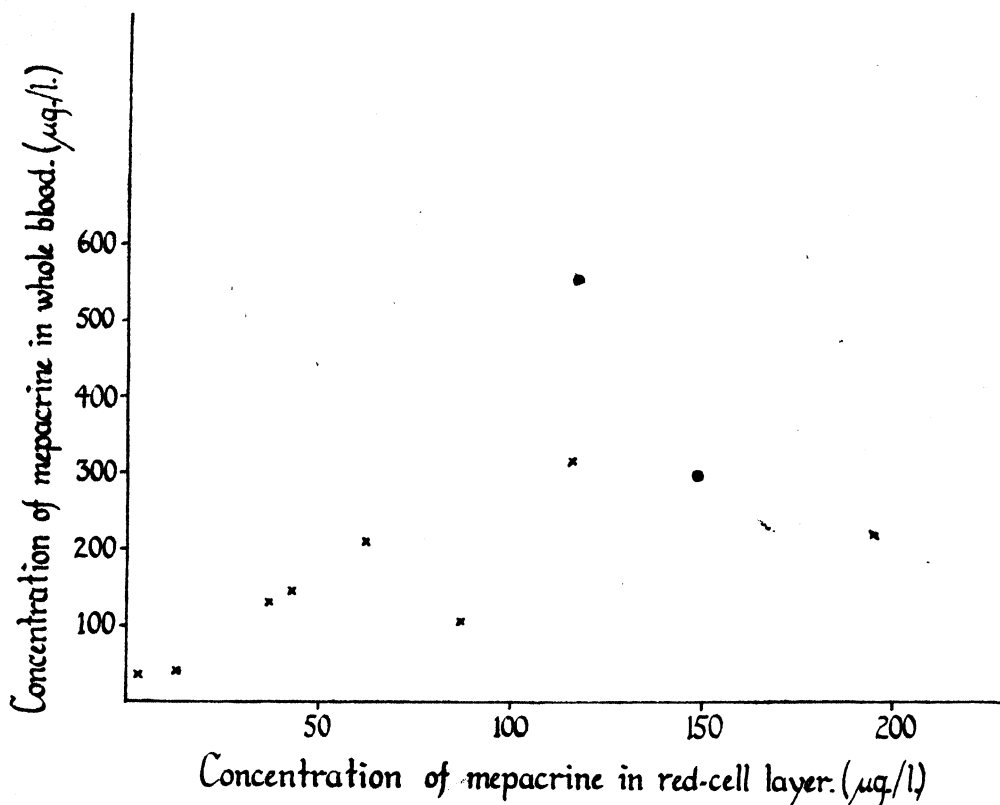


FIG. 2. Relation between concentration of mepacrine in whole blood and in red-cell layer.
 × = Observed values; ● = Values of Shannon *et al.* (1944).

The concentration of mepacrine in the plasma was plotted against the concentration in the red-cell layer (fig. 1), as was the concentration of mepacrine in the whole blood (fig. 2). An inspection of the graphs will show that there is a closer correlation between the plasma concentration and the red-cell concentration than between the whole-blood concentration and the red-cell concentration. The coefficient of regression of the red-cell concentration on the whole-blood concentration was shown to be 0.250 ± 0.108 , whereas that of the red-cell concentration on the plasma concentration was 1.613 ± 0.181 . It has also been shown that the regression equation for the relation between the red-cell concentration and the plasma concentration ($y = 1.613x + 8.0$, where y = the concentration of mepacrine in the red cells and x = the concentration of mepacrine in the plasma) gives a significantly better estimate of the concentration of mepacrine in the red cells than

does the regression equation of the relation between the red-cell concentration and the whole-blood concentration ($y = 0.250x + 29.3$, where y = the concentration of mepacrine in the red cells and x = the concentration of mepacrine in the whole blood).

For the data available and over the range of mepacrine concentrations considered, the chance of this observation being fortuitous is less than 1 in 100. It therefore follows that, if the concentration of mepacrine in the red cells is required, plasma determinations are of more value than those on whole blood.

As will be seen in fig. 3, there is a high correlation between the concentration of mepacrine in the whole blood and the concentration of the drug in the white-cell layer.

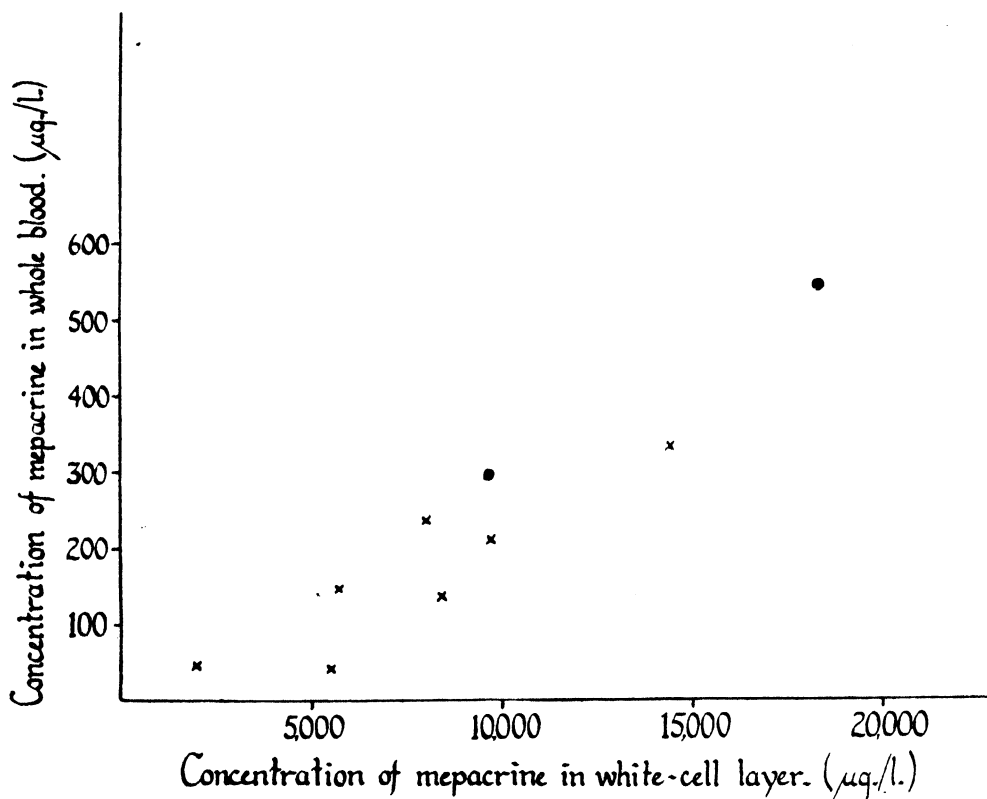


FIG. 3. Relation between concentration of mepacrine in whole blood and in white-cell layer.
 x = Observed values; ● = Values of Shannon *et al.* (1944).

The coefficient of regression of the white-cell concentration on the whole-blood concentration is 28.9 ± 1.2 . Again it has been found that the regression equation for the relation between the white-cell layer concentration and the whole-blood concentration gives a significantly better estimate of the whole-blood concentration than does the regression equation for the relation between the red-cell concentration and the whole-blood concentration ($y = 28.9x + 2,600$, where y = concentration of mepacrine in the white-cell layer and x = concentration of mepacrine in whole blood). This means that the mepacrine in the white-cell layer contributes more to the whole-blood concentration than does the mepacrine in the red-cell layer.

MEPACRINE CONCENTRATION IN BLOOD AND PLASMA OF PATIENTS WITH MYELOID LEUKAEMIA

Two patients suffering from myeloid leukaemia were given mepacrine 0.1 gm. daily by mouth, and the concentration of the drug was determined in the blood and plasma.

CASE 1

Female, aged 44, under the care of Professor L. J. Witts, Radcliffe Infirmary, Oxford.

The patient had had chronic myeloid leukaemia for several years. She returned to hospital for further X-ray treatment. She was given 0.1 gm. mepacrine, and this dose was repeated daily for 14 days, when the drug was discontinued. Blood samples were withdrawn for mepacrine estimations on whole blood and plasma, as shown in Table IV. The white blood-cell count was 160,000 per c.mm. at the commencement of the experiment and 264,000 per c.mm. 17 days later.

TABLE IV
Concentration of mepacrine in blood and plasma of Patient 1

Date	Day of dosage	Mepacrine concentration (in $\mu\text{gm./l.}$)		White-cell count (cells/c.mm.)
		Blood	Plasma	
18.12.43	1	250	10	160,200 (primitive cells, 62%; polymorphs, 36.5%)
19.12.43	2	240	20	
21.12.43	4	1,090	20	261,500 (primitive cells, 51%; polymorphs, 48%)
24.12.43	7	2,720	75	
26.12.43	9*	3,340	85	
30.12.43	13	5,040	60	
3. 1.44	17	2,540	20	249,500
6. 1.44	20	1,060	10	264,500

* Last day on drug.

CASE 2

Male, aged 25, under the care of Professor L. J. Witts, Radcliffe Infirmary, Oxford.

The patient had acute myeloid leukaemia of several weeks' duration. He was given 0.1 gm. mepacrine on two consecutive days. The patient died the following day, estimations for that day being done on a post-mortem blood sample. The white blood-cell count was 334,000 per c.mm. on the second day and 327,000 per c.mm. on the day of death.

Blood samples were withdrawn on the second day for estimation of mepacrine in whole blood and plasma (Table V).

TABLE V
Concentration of mepacrine in blood and plasma of Patient 2

Date	Day of dosage	Mepacrine concentration (in $\mu\text{gm./l.}$)		White-cell count (cells/c.mm.)
		Blood	Plasma	
29.12.43	1	590	65	334,000
30.12.43	2	705	415*	327,000

* Estimation done on blood post mortem.

It will be seen from Tables IV and V that the plasma concentrations were of the same order as those of normal persons receiving the same dose of the drug. The concentration of the drug in the whole blood was in striking contrast. Whereas normally the concentration of the drug in the blood of patients who have received 0.1 gm. daily for 13 days is of the order of 150 $\mu\text{gm./l.}$, Patient 1 had a concentration of 5,000 $\mu\text{gm./l.}$ —over 30 times as much—and Patient 2, after having received only 0.2 gm. of the drug, had a blood concentration of over 700 $\mu\text{gm./l.}$ These abnormally high blood concentrations

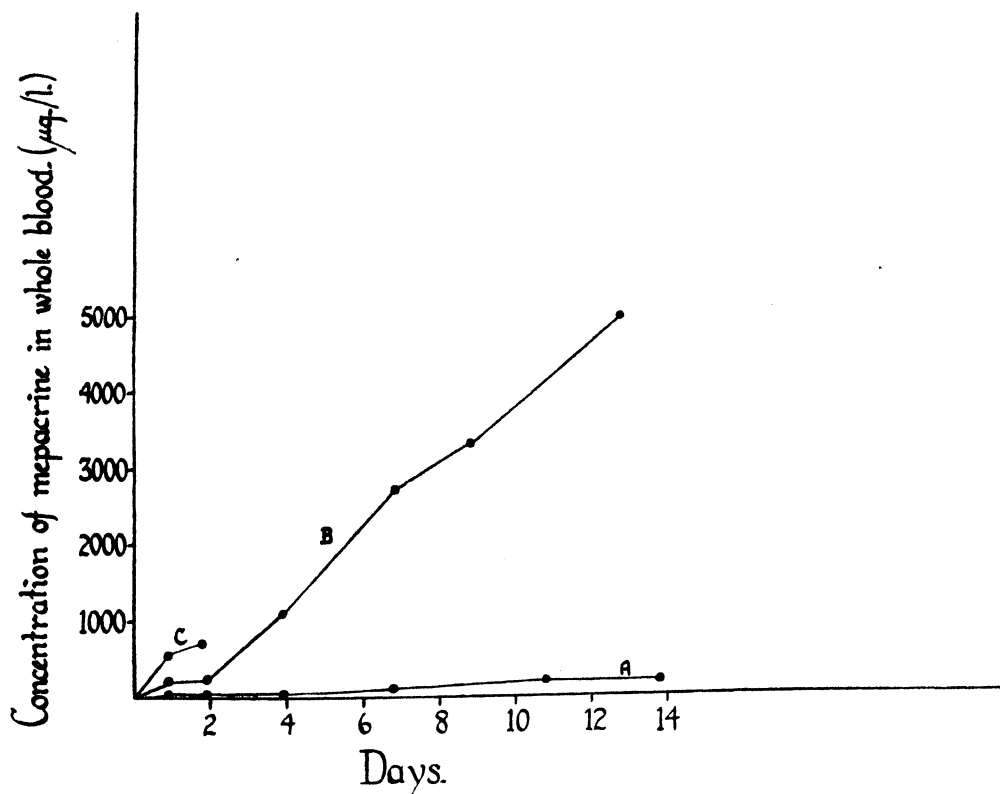


FIG. 4. Mepacrine concentration in whole blood ($\mu\text{gm./litre}$) of two patients suffering from myeloid leukaemia (B and C) and of normal undergraduates (A; mean of eight).

are undoubtedly due to the increased white-cell counts. The above points are well illustrated in fig. 4. Since mepacrine-recovery experiments indicate that 100 per cent. recovery is less readily obtained from leukaemic blood than from blood obtained from normal persons, the estimates of the mepacrine content of the leukaemic blood given in the tables are, if anything, on the low side.

DISCUSSION

The observation that the concentration of mepacrine in plasma gives an estimate of the concentration of the drug in red cells is not surprising, in view of the *in vitro* observations of Shannon *et al.* (1944), who found that red cells equilibrated with mepacrine-containing plasma took up a fixed proportion of the drug. The ratio of drug concentration

in red cells to that in plasma observed by these authors was 2.1-2.4 to 1. The average ratio in the series reported here was 1.6 to 1. It must be remembered, however, that Shannon's original observations were made with dog blood and that the conditions (equilibration was done at 22° C.) were not physiological.

It has thus been shown that, if the white-cell count is normal, up to 80 per cent. of the mepacrine contained in the blood is in the white-cell layer. If the white-cell count is abnormally high, as in the case of patients with leukaemia, the percentage of mepacrine in the white cells will be considerably higher than this figure. It is possible that much of the mepacrine in the white-cell layer is not in the white cells themselves but in the platelets. Whether this is so can only be determined by further experiment, although preliminary observations by us on patients with high platelet counts and thrombocytopenic purpura would indicate that this is not the case. What does seem clear is that the greater portion of the blood mepacrine is neither in the plasma nor in the red cells. For this reason we agree with the statement of Shannon *et al.* (1944) that 'the whole blood atabrine concentration is more apt to reflect the change in the leucocyte count than the underlying plasma atabrine concentration.'

It is not yet known whether suppression is best obtained by high peaks of maximum mepacrine concentration in body fluids, or by preventing the concentration in the body fluids from falling below a minimum during the 24-hour period, or by the degree of saturation of the tissues. If it is the concentration of mepacrine in the red cells (either maximum or minimum over a 24-hour period) which is important, mepacrine estimations on plasma are of more value than those on whole blood, since red-cell concentration follows more closely the plasma concentration than whole-blood concentration. On theoretical grounds, the concentration of the unbound mepacrine in the plasma should give a more accurate estimate of the mepacrine in the red cells than the total concentration of mepacrine in plasma, but from a practical point of view this estimation would be difficult to perform. Nevertheless, it seems reasonable to assume that, for the normal plasma protein values, the percentage of the drug bound by the protein will be constant.

It has been demonstrated repeatedly that the mepacrine concentration in serum is significantly higher than that in plasma from the same blood sample. This indicates that some of the drug is liberated from the white cells or platelets into the serum. Estimations in serum are, therefore, unreliable. Technically, the estimation of mepacrine in plasma is more difficult. We have confirmed the observation of Shannon *et al.* (1944) that, when an oxalated specimen of the blood is allowed to stand, mepacrine reverts from the cells to the plasma. It therefore follows that blood must be centrifuged *immediately* it is taken. It is our practice to centrifuge the blood at 1,500 revolutions per minute for 30 minutes in the ward within 10 minutes of its withdrawal, to pipette off the supernatant plasma with a teat pipette, and to transport the plasma as soon as possible to the laboratory, where it is recentrifuged at the same or at a higher rate for a further 30 minutes to one hour. It has been found that the mepacrine in the recentrifuged plasma is much more stable than that in the whole blood. Preliminary experiments have shown that in whole blood, possibly in the white cells, there is an agent capable of slowly destroying the drug. Another point which must be borne in mind is that the concentration of the drug in plasma is much lower than that in whole blood, and is usually of such an order that it cannot be estimated as accurately (Army Malaria Research Unit, 1946).

SUMMARY

1. The observation of Shannon *et al.* (1944), that the concentration of mepacrine in the white-cell layer is greater than that in either the plasma or the red-cell layer, has been confirmed.

2. With normal white-cell counts, up to 80 per cent. of the mepacrine in whole blood is in the white-cell layer.

3. The concentration of mepacrine in the plasma gives a better estimate of the concentration of mepacrine in the red cells than does the concentration of mepacrine in whole blood.

4. The concentration of mepacrine in the white-cell layer gives a significantly better estimate of the whole-blood concentration than does the concentration of mepacrine in the red-cell layer.

5. The concentration of mepacrine in the blood of leukaemic patients who have received the drug by mouth is much higher than that in the blood of normal persons who have received the same dose, whereas that in the plasma is of the same order.

6. These results are discussed in relation to the problem of estimating mepacrine in whole blood and/or plasma.

7. It is concluded that, if it is desired to know the concentration of mepacrine in the red cells, the concentration in the plasma gives a better estimate than that in the whole blood.

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SUPPRESSION OF BENIGN TERTIAN MALARIA WITH MEPACRINE: AN INVESTIGATION OF 247 CASES OF APPARENT FAILURE

BY

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The great value of mepacrine in suppressing malaria under Service conditions in different theatres of operations was amply proven during the war and has been widely commented upon (Covell, 1943; Findlay and Stevenson, 1944; McCoy, 1945; and Sinton, 1945). However, it has never been completely effective under field conditions, and therefore, in 1944, the First Field Section, Malaria Research Unit, was sent to Italy to study the use of mepacrine in the suppression and treatment of benign tertian malaria. It had at its disposal the techniques necessary to measure the amounts of mepacrine in plasma and other biological fluids, and was thus able to study the absorption and metabolism of the drug and to relate suppressive and therapeutic results to some of the events which follow its ingestion. This paper reports the results of an examination of a series of cases of benign tertian malaria for the causes of the apparent failure of mepacrine suppression. The conclusion was that the failure was more often apparent than real.

CASE MATERIAL

The 247 patients studied were admitted to two base hospitals between September 13th and November 15th, 1944. The greater part of the area drained by these hospitals was only slightly endemic, but it also contained regions which were highly malarious and regions which were non-malarious. Except for a few small units which were poorly sited from the hygienic point of view, the risk of malarial infection was not great. The entire area, however, had been designated a 'mepacrine area' and the men in all units were supposed to be taking mepacrine 0.1 gm. per day until November 15th. In 117 cases it was the man's first clinical attack of malaria; 100 were suffering from the second to fourth attack; 18 from the fifth to seventh attack; and 13 were in their eighth or subsequent attack.

USE OF SUPPRESSIVE MEPACRINE

On admission to hospital each man was questioned regarding the regularity with which he had taken suppressive doses of mepacrine. Only 27 per cent. (66/247) claimed to have taken the full suppressive dose of one tablet (0.1 gm.) daily. Without doubt many of these men were making false claims, and the actual number who were taking mepacrine regularly was certainly much smaller. There are two reasons in our own evidence for believing this to be so. The results of questioning varied with the interrogator, and one of us found that, on the average, the group of men whom he questioned

answered that they had taken less mepacrine than the group questioned by the other—which suggests that the answers received by both were in part untruthful. Also, when the plasma mepacrine concentrations found in the men who claimed to have taken seven tablets a week were compared with the plasma mepacrine concentrations found in a control-group of officers and men known to be taking mepacrine with almost perfect

TABLE I
Suppressive mepacrine taken by 247 men admitted to hospital with malaria :
summary of men's statements

	No. of cases	Average plasma mepacrine, $\mu\text{gm.}/1,000 \text{ ml.}$
Took 7 tablets a week	66 (a)	9.07
Took 5-6 tablets a week	24	10.08
Took 2-4 tablets a week	37	5.70
Took mepacrine 'irregularly'	17	7.40
Took an increased dose during 7 days before admission	14 (b)	16.15
Stopped taking mepacrine 1 week before admission	18 (c)	5.37
Stopped taking mepacrine 2 weeks before admission	17 (d)	5.14
Stopped taking mepacrine 3-4 weeks before admission	21 (e)	6.45
Stopped taking mepacrine 5-6 weeks before admission	5	4.40
Took no mepacrine	24	—
No information	4	10.25
Total	247	

(a) Plasma mepacrine estimated in 64 cases.

(b) Plasma mepacrine estimated in 13 cases.

(c) Plasma mepacrine estimated in 16 cases.

(d) Plasma mepacrine estimated in 14 cases.

(e) Plasma mepacrine estimated in 19 cases.

TABLE II
Relation between previous malarial attacks and the use of suppressive mepacrine

No. of attacks of malaria	Use of mepacrine : no. of patients who			
	Took 7 tablets a week	Used mepacrine irregularly	Used no mepacrine	Took an increased dose in 7 days prior to admission
Initial attack	31	59	18	6
Second or later attack	35	80	6	8

regularity, it was found that only two-thirds of them (41/64) had plasma-levels which were within the range of levels found in the control-group (see below). These considerations, when taken with the results of well-disciplined control experiments (Fairley, 1945), suggest that the number of men who had actually taken mepacrine regularly and who could be considered genuine 'mepacrine break-throughs' was in fact very small,

A large proportion of the cases (78/247) were men who admitted that they had taken mepacrine irregularly. Another large group (61/247) had stopped taking mepacrine one to six weeks before admission, and 24 had not taken mepacrine for more than six weeks.

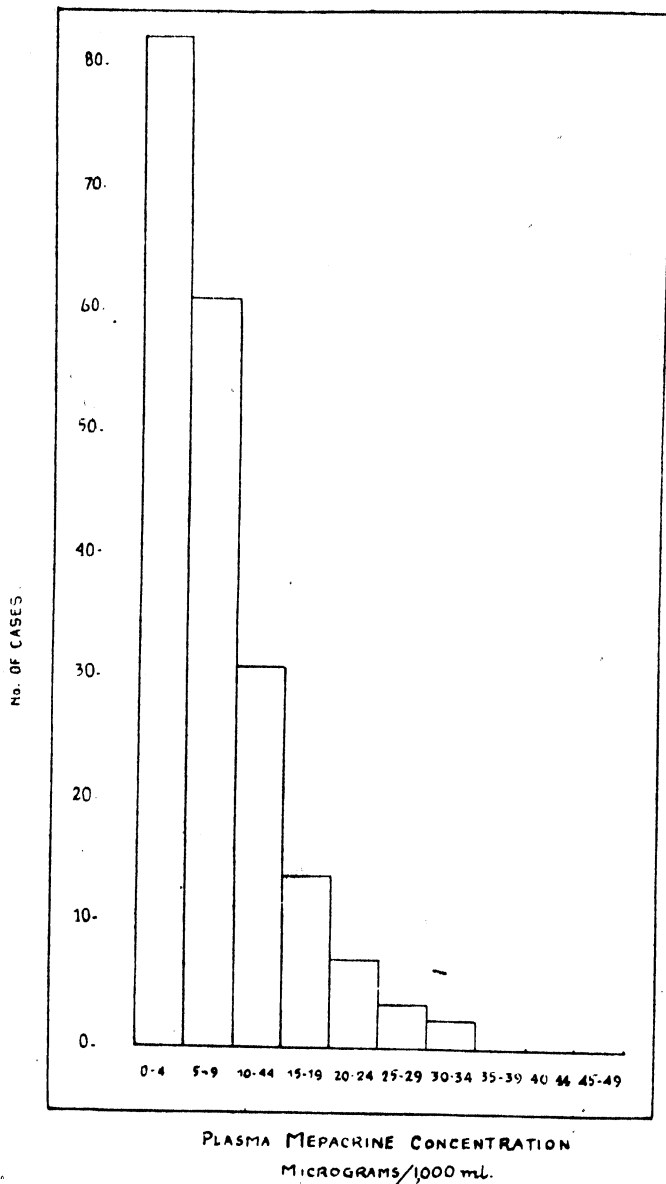


FIG. 1. Plasma mepacrine concentrations in 200 cases of benign tertian malaria at the time of admission to hospital.

When the cases were grouped according to the number of previous attacks of malaria it was found that previous attacks did not apparently increase a man's willingness to take mepacrine regularly, though it did tend to make him take some. (Value of t when the

t-test is applied to the difference between the numbers of men taking and those not taking mepacrine in the groups (a) initial attacks, (b) second or subsequent attacks, is 3.17; $P < 0.05$.)

PLASMA MEPACRINE CONCENTRATIONS

In 215 cases specimens of plasma were obtained for estimation of the plasma mepacrine concentration. In 14, a dose greater than one tablet a day had been taken during the previous week, and in one case the specimen was taken less than 24 hours after the last tablet; these cases have been excluded from the group of 200 cases discussed in the next paragraph. In eight cases who had been on mepacrine a specimen was not taken or was lost in the centrifuge. All the estimations were done by a modification of the photofluoroscopic method of Masen (1943). The standard deviation of the difference between duplicate estimations on known solutions was less than 2 $\mu\text{gm.}/1,000\text{ ml.}$

In the 200 cases in which minimal plasma mepacrine concentrations* were determined the mean concentration was 7.5 $\mu\text{gm.}/1,000\text{ ml.}$, S.E. 0.45. The modal value was 2 $\mu\text{gm.}/1,000\text{ ml.}$ and the first and third quartiles were 2 and 11 $\mu\text{gm.}/1,000\text{ ml.}$ respectively (fig. 1).

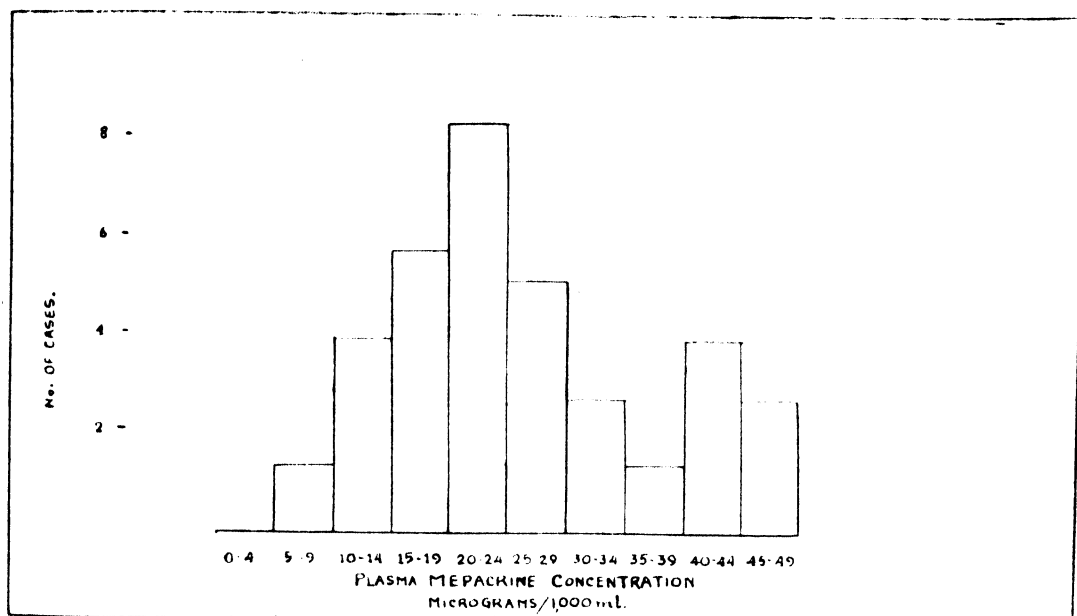


FIG. 2. Plasma mepacrine concentrations in 29 men and women who had been taking mepacrine 0.1 gm. per day for 5-6 months.

The levels in this group which was supposed to be taking one tablet of mepacrine a day should be compared with the levels found in a control-group drawn from medical officers and nursing officers of the Royal Canadian Army Medical Corps and from the

* By the term 'minimal plasma mepacrine concentration' is meant the plasma mepacrine concentration 24 hours after taking the last suppressive dose.

personnel of a Malaria Field Laboratory. These officers and other ranks had been on mepacrine for at least five months and stated that they had taken mepacrine with almost complete regularity; the other ranks had taken their tablets on parade. The mean level in this group was $25.3 \mu\text{gm./1,000 ml.}$, S.E. 1.92, and the levels ranged from 8 to 48 $\mu\text{gm./1,000 ml.}$ (fig. 2).

The comparatively slight risk of malarial infection and the laxness of mepacrine discipline in the area caused some to suggest that the plasma mepacrine concentrations in our series of malarial cases might be of the same order in a non-malarial group from the same area, and might perhaps bear no relation to the onset of their malarial attack. For this reason plasma specimens were obtained from a series of medical cases admitted contemporaneously with the malarial cases. A consecutive series of 36 cases was collected in which there was a definite diagnosis other than malaria, and in these the mean plasma mepacrine concentration was $10.5 \mu\text{gm./1,000 ml.}$, S.E. 1.15. This mean is significantly different from the mean of $7.5 \mu\text{gm./1,000 ml.}$, S.E. 0.45, found in the series of malarial cases ($t = 2.50$, $P < 0.02$), and the suggestion is therefore not upheld (Table III). A

TABLE III

Average plasma mepacrine concentrations in (a) 200 cases of malaria, (b) 36 control medical cases in which there was a definite diagnosis other than malaria

	Average plasma mepacrine concentrations, $\mu\text{gm./1,000 ml.}$
Malaria cases	7.50, S.E. 0.45
Control cases	10.58, S.E. 1.15

small number of cases was admitted from the Anzio area, where malaria was epidemic among the civilian population and the risk of infection was great, and a comparison of these cases with control-cases admitted from the same area gives a more emphatic proof that the protection afforded by mepacrine is related to the plasma mepacrine concentration (Table IV). Here the control-group had a mean plasma mepacrine concentration of $17.6 \mu\text{gm./1,000 ml.}$ as compared with $8.7 \mu\text{gm./1,000 ml.}$ in the malarial group ($t = 4.83$, $P < 0.02$).

TABLE IV

Average plasma mepacrine concentrations in (a) 15 cases of benign tertian malaria, (b) 9 cases of medical diseases other than malaria from an epidemic area

	Average plasma mepacrine concentrations, $\mu\text{gm./1,000 ml.}$
Malaria cases	8.7, S.E. 1.40
Control cases	17.6, S.E. 3.80

The reason why plasma mepacrine concentrations greater than $15 \mu\text{gm./1,000 ml.}$ —concentrations which are apparently protective in the great majority of men—did not prevent an attack of malaria in 28 of our cases is not clear, though several can be suggested.

In four cases the drug had been stopped a short time previously, and had probably been taken irregularly before that. In two cases the men admitted that they had taken their tablets irregularly, and the plasma mepacrine concentration when they were admitted to hospital was not necessarily their average minimal plasma mepacrine concentration during the previous weeks. Some men in the series admitted taking increased doses when premonitory symptoms appeared, and there were probably some who had done so but did not admit it. It would not be unreasonable to expect a few cases in which those other factors, which combine with the protection afforded by an average plasma mepacrine concentration, were so small, because of special circumstances, that the individual's resistance to the infection was overcome; but in no case were we able to find evidence of any other illness or condition which might have lowered general resistance, and it was our opinion that in the majority of cases a lack of veracity had contributed to the anomalous result. It is a possibility that in a few cases we were dealing with a strain of the parasite which was unusually resistant to mepacrine, because some of them had plasma concentrations which in other persons had a therapeutic effect. However, it was not considered a probability, because the response to therapeutic doses of mepacrine was the same as in other cases in the series.

Plasma mepacrine concentrations were measured in 74 cases in which the men claimed that they had been taking regularly 6-7 tablets each week. In 36 cases the patients were having their first attack of malaria, and they had a mean plasma mepacrine concentration of 11.2 $\mu\text{gm.}/1,000 \text{ ml.}$, S.E. 1.49. In 38 cases the patients were having their second or subsequent attack (whether relapse or reinfection it was impossible to determine in the circumstances), and they had a mean plasma mepacrine concentration of 6.7 $\mu\text{gm.}/1,000 \text{ ml.}$, S.E. 1.15 ($t = 2.253$, $P < 0.05$). If the men really had been taking 6-7 tablets a week this may mean that, on the average, men who have had previous attacks of malaria 'break through' at a lower level than men who have not, and it may mean that men who have multiple attacks do not, on the average, have as high plasma mepacrine concentrations as do other men. Evidence will be presented in another paper which suggests that men who have multiple attacks of malaria have plasma mepacrine concentrations while receiving therapeutic doses of mepacrine which are lower than the average. There were 25 cases in which the present attack was at least the patient's sixth; some had taken mepacrine regularly, some irregularly. The mean plasma mepacrine concentration in these men was 5.4 $\mu\text{gm.}/1,000 \text{ ml.}$, S.E. 1.08.

An additional 16 cases of malaria were obtained from a forward area where conditions were much more rigorous than in the base area where the Field Section was stationed. The average plasma mepacrine concentration in these cases was 3.7 $\mu\text{gm.}/1,000 \text{ ml.}$ (range 0-9), which is evidence against the suggestion that the very rigorous conditions in which these men were living might lead to 'break-throughs' at higher plasma mepacrine concentrations than were seen in men living in more ordinary surroundings.

EXCRETION OF MEPACRINE IN THE URINE

The estimation of mepacrine in the urine at the time of admission to hospital showed that the low plasma-levels found in most of the men who claimed that they had taken 6-7 tablets a week were not to be explained by an unduly large excretion of the drug by the kidneys. In 41 cases a two-hour specimen of urine was collected immediately before blood was withdrawn for the estimation of plasma mepacrine. In the 17 cases of this

group in which the men claimed to have taken 6-7 tablets a week, the average amount of mepacrine excreted in a two-hour period was 0.091 mgm., and the average plasma mepacrine concentration was 7.8 μ gm./1,000 ml. The corresponding figures in a group of 24 men who said that they had been taking less than six tablets a week were 0.063 mgm. and 8.2 μ gm./1,000 ml. The difference between the two average amounts of mepacrine excreted is not significant, and in neither case was it a sufficient amount in relation to the daily dose of mepacrine materially to affect the plasma-level.

DISCUSSION

The purpose of this investigation was to find why there were cases of malaria in a population in which, in theory, every man exposed to infection was taking a daily dose of mepacrine which suppressed malaria below the clinical level in the great majority. The first step was to show that there is a relation between the plasma mepacrine concentration and the protection afforded against malaria. That this is probably so was established by the observations in the malarial cases and in the control-groups. The group of malarial cases in which usually adequate plasma mepacrine concentrations (15 μ gm./1,000 ml. and upwards) were found might seem to be evidence against such a relation, but it was possible to show, by obtaining a history of irregular drug-taking, that in a number the plasma mepacrine concentration at the time of admission to hospital was not necessarily the average minimal plasma mepacrine concentration during the previous two weeks, and it was felt that, if complete veracity had prevailed, more of the results in this group would have been explained in the same way. The problem, then, was the discovery of the causes of the low plasma mepacrine concentrations found in the cases of malaria.

In a group of men supposed to be on a suppressive mepacrine régime, low plasma mepacrine concentrations may occur as a result of one or more of the following factors :

1. Failure to take the drug regularly.
2. Poor absorption from the gut.
3. Abnormal excretion in the urine.
4. Abnormal partition of the drug between the plasma and the tissues.
5. Abnormally rapid destruction of the drug.

By the admission of the patients themselves, failure to take mepacrine regularly was the factor involved in 73 per cent. of our cases, and the real size of the group was probably larger. In only two of our cases did we have evidence from someone other than the patient that the use of mepacrine had been satisfactory ; for the rest, their word had to be taken. That an abnormal excretion of the drug in the urine was not the cause of the low levels was shown by the measurement of the urinary mepacrine in those who claimed that they had been taking six or seven tablets per week. Other experiments, which will be reported in another paper, were carried out on patients receiving therapeutic doses, and these showed that poor absorption from the gut and abnormal partition between the tissues and the plasma were not causes of relatively low concentrations in the plasma. By exclusion, the causative factor of low plasma mepacrine concentrations in persons who take mepacrine regularly would seem to be an abnormally rapid destruction of the drug.

The conclusion that the important cause of the apparent failure of mepacrine to suppress malaria was carelessness in taking the drug is at variance with the findings of Findlay and Stevenson (1944) in West Africa, who considered that failure to take mepacrine

accounted for less than a third of the apparent failures. Bomford (1944), in the same theatre, also concluded that attacks of malaria were not related to obvious ill-discipline. However, the results of the experiments at the Australian Medical Research Unit, in which mepacrine 0.1 gm. per day was found completely to suppress benign tertian malaria when drug administration was rigidly supervised (Fairley, 1945), support our conclusion. Missiroli (1945), working with a civilian population, has also found that when mepacrine is given under adequate supervision almost 100 per cent. protection is obtained. It is clear that the efficiency of mepacrine suppression can be improved more by increasing the regularity of its use than by finding how to use the drug in those few who metabolize it abnormally.

A full description of the reasons why many men were not using mepacrine regularly in the Central Mediterranean Force in 1944 would be outside the scope of this paper, but some of our observations may be noted here. The alleged side-effects of mepacrine were seldom a reason. Much more important was a lack of knowledge of the purpose and of the great efficiency of mepacrine suppression, and a resultant lack of enthusiasm in enforcing its use. The rôle of mepacrine is a difficult concept for the lay officer to grasp. He is unable to see its value when it so often apparently fails to suppress malaria, and when the cessation of mepacrine administration is followed by so many relapses. The first of these objections can now be shown to arise usually from his own shortcomings; but, to meet the second, well-planned propaganda is needed whenever mepacrine is used, to remove the misapprehensions which diminish the value of the drug in his eyes and which thereby operate against the maintenance of good mepacrine discipline. The administration of mepacrine must be the responsibility of the officers. A measure which must be used daily, often when the cost of neglect appears to the partially informed to be negligible, cannot be left to the men themselves except with unfortunate results in proportion to the risk of malarial infection. Officers and senior N.C.O.s must see not only that each man is issued with a tablet, but also that he swallows it. Sir Thomas Legge's second axiom for the prevention of industrial accidents was this: 'If you can bring an influence to bear external to the workman (i.e., one over which he can exercise no control) you will be successful; and if you cannot or do not, you will never be wholly successful.'

It is an axiom which applies equally well to preventive tropical medicine, and, for the suppression of malaria with mepacrine to be as successful as it should be, the taking of mepacrine must be under an external influence.

SUMMARY

1. A series of 247 cases of benign tertian malaria seen in Italy in 1944 was investigated in an attempt to find why mepacrine suppression had not been completely successful.
2. Seventy-three per cent. of the patients admitted that they had not taken regularly the suppressive dose of mepacrine 0.1 gm. per day.
3. The mean plasma mepacrine concentration was $7.5 \mu\text{gm./1,000 ml.}$, S.E. 0.45, in 200 cases in which it was measured. In a control-group of healthy persons known to have received the full suppressive dose, the mean plasma mepacrine concentration was $25.3 \mu\text{gm./1,000 ml.}$, S.E. 1.92.
4. Measurement of the excretion of mepacrine in the urine showed that the low concentrations in the plasma of cases of malaria were not due to an abnormal excretion by the kidneys.

5. Failure to use mepacrine regularly was a much more important cause of apparent failure of suppression than was abnormal metabolism of the drug.

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CARBOHYDRATE METABOLISM IN BOVINE TRYPANOSOMIASIS

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INTRODUCTION

It has been recognized for a considerable time that trypanosome infections can produce a marked disturbance in the carbohydrate metabolism of the host species. The majority of these previous investigations, however, have been carried out in experimental infections of small laboratory animals, when the disease set up does not simulate that produced in the natural host. With the single exception of Wormall's (1932) investigations, previous work on this aspect of trypanosomiasis has been confined to random observations regarding blood-sugar, alkali reserve and blood lactic acid, the results obtained by different workers being, on the whole, at considerable variance and inconclusive. In general it is agreed that there is no progressive hypoglycaemia, and that any initial hyperglycaemia is finally replaced by a marked pre-mortal or crisis hypoglycaemia associated with depleted alkali reserve and increased blood lactic acid.

French (1938) gives a comprehensive summary of previous work on carbohydrate metabolism in animal trypanosomiasis, to which readers are referred for full details, and concludes from his own experiments that there is a marked disturbance in *Trypanosoma congolense* and *T. brucei* infections in the larger domestic animals. Hudson (1944) describes cases of trypanosomiasis in cattle caused by *T. vivax* or by mixed infections of *T. congolense* and *T. vivax*, in which the disease follows an acute course like that produced in pigs by *T. simiae*. In these acute cases of trypanosomiasis in both cattle and swine, death is produced in a few days and is associated with a marked hypoglycaemia.

It is noticeable that, in previous investigations of the carbohydrate metabolism during trypanosomiasis other than those of Wormall (1932), no attempts have been made to investigate the disease by means of functional tests. Wormall (1932) investigated *T. gambiense* infections in man by means of oral glucose-tolerance tests and concluded that there was no serious impairment of the capacity of the liver to deal with glucose in the early stages of trypanosomiasis.

Following the elaboration of a suitable intravenous glucose-tolerance test for bovines by Bell and Jones (1945), in which it was shown that the tolerance of this species for glucose is comparable with that of others, it was decided to apply the test to *T. congolense* infections, in an attempt to prove whether any real dysfunction of carbohydrate metabolism is, in fact, caused by this disease. It was planned originally to make comprehensive investigations on these lines throughout the course of infection, but, for reasons beyond our control, the work had to be limited to a study of the disease in the chronic stage.

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TABLE I
Blood constituents of 22 bovines infected with *T. congolense* and of four controls, at time of tests

No.	Initial or max. weight (lb.)	Max. weight-loss* (lb.)	Weight at test (lb.)	Red blood-cells (million)	Hb. gm. %	Packed-cell volume %	Mean corpuscular volume (c.μ)	Mean corpuscular haemoglobin (γγ)	Mean corpuscular haemoglobin concentration %	Plasma CO ₂ -C.P. (volumes %)	Mgm./100 ml. blood	
											Glucose	NaCl
5313	679	137	623	7.4	9.4	32	43.3	12.7	29.4	62.6	70.7	453.8
5314	552	104	526	7.1	9.1	32	45.1	12.8	28.4	57.6	80.6	478.5
5316	649	161	531	7.1	9.1	30	42.3	12.8	30.3	64.5	54.2	462.0
5317	528	100	504	5.3	8.2	27	51.0	15.5	28.3	59.8	57.3	453.8
5318	582	98	588	7.5	10.2	36	48.1	13.6	28.3	56.0	60.2	445.5
5319	513	92	452	7.0	9.2	32	45.7	13.1	28.8	55.1	74.3	478.5
5320	539	71	545	5.4	8.0	27	50.0	14.8	29.6	62.6	69.7	478.5
5322	505	85	494	7.7	10.2	36	46.7	13.2	28.4	61.7	69.9	445.5
5323	528	57	532	7.6	11.1	37	48.7	14.6	30.0	60.7	80.0	437.3
5324	575	47	584	6.6	10.1	34	51.6	15.3	29.7	64.5	69.4	470.3
5325	528	102	530	7.7	9.1	32	41.5	11.8	28.4	74.0	73.0	387.8
5327	549	77	542	6.2	10.1	32	51.7	16.3	31.6	61.7	63.5	420.8
5328	477	71	498	7.5	9.5	34	45.4	12.7	28.0	61.7	53.0	453.8
5329	515	107	502	8.2	10.6	38	46.5	12.9	27.9	58.8	76.3	478.5
5331	526	106	507	8.5	9.0	29	34.2	10.6	31.0	62.6	61.2	478.5
5332	561	197	476	6.0	8.3	29	48.3	13.8	28.6	60.7	59.2	462.0
5333	548	58	572	7.3	10.5	34	46.6	14.4	30.9	57.0	49.7	437.3
5335	536	116	448	4.5	6.8	24	53.3	15.1	28.3	62.6	64.3	478.5
5336	461	77	455	8.5	9.6	32	37.6	11.3	30.0	58.9	90.5	453.8
5337	517	33	549	9.1	10.6	35	38.5	11.6	30.3	61.7	67.8	462.0
5338	522	98	476	6.4	7.7	26	40.7	12.0	29.6	60.7	85.1	470.3
5339	431	37	448	6.8	8.9	30	44.2	13.1	29.7	61.7	97.6	453.8
Extreme range	431/679	33/197	448/623	4.5/9.1	6.8/11.1	24/38	34.2/53.3	10.6/16.3	27.9/31.6	55.1/74.0	49.7/97.6	387.8/478.5
Mean	537	92	520	7.1	9.3	32	45.5	13.4	29.5	61.2	69.4	456.4
4988	584	+20	604	7.2	9.7	—	—	13.5	—	61.7	49.8	470.3
5274	300	+14	314	7.2	8.4	—	—	11.7	—	64.5	52.3	470.3
5294	289	3	286	8.0	9.6	—	—	12.0	—	64.5	52.8	462.0
5298	309	+13	322	7.6	11.8	—	—	15.5	—	67.3	52.3	453.8

* Throughout the period of infection.

EXPERIMENTAL

Twenty-six adult Zebu cattle were infected by inoculation of pooled blood from four bovines previously infected by *Glossina morsitans* with the 'Kikaya' and 'Buruli' strains of *T. congolense*, and nine days later with citrated blood from mice infected with the 'Kayonza' strain. Of this group of animals four died of *T. congolense* infection within three months; the remainder recovered after crisis and passed into the chronic stages of the disease. The 22 survivors were submitted to the intravenous glucose-tolerance tests approximately seven months after infection, and no further deaths have occurred in these chronic infections (i.e., about one year after infection).

TECHNIQUE

The methods used for both blood and urine analysis were the same as those previously described by the present authors (1945), as also was the technique of the intravenous injection, in which a constant dose of 0.2 gm./kgm. glucose was injected at a uniform rate of 20 c.cm. per minute using a 50 per cent. solution of anhydrous glucose. Control glucose estimations by the Folin and Wu method on aqueous solutions of pure dextrose ranging from 76 to 242 mgm. per cent. gave recoveries varying from 92.3 to 108 per cent.

RESULTS

1. Blood Physical and Chemical Findings

Clinically the animals showed no abnormality and were all in good condition, weight-decreases, on the average, having returned to normal at the time of the tests. All showed trypanosomes in the peripheral blood almost to the time of the investigation. Examination of the blood physical findings recorded in Table I, however, shows that they had all recovered from the anaemic crisis in varying degrees, as evidenced by the relative increase in the erythrocyte count, haemoglobin and packed-cell volume, and by the approximately normal mean corpuscular haemoglobin, compared with previous findings in this disease.

Fig. 1 shows the level of these constituents in relation to the normal range for healthy Ankole and Zebu stock established at this laboratory. It will be seen that the erythrocyte count and haemoglobin were well on the way to approaching normal, though the packed-cell volume still remained slightly subnormal. The average mean corpuscular volume of 45.5 c. μ is at the upper limit of the normal range, but it will be seen from fig. 1 that over half the values obtained were above this level. The mean corpuscular haemoglobin concentration had fallen from the higher levels previously encountered in this disease to values slightly below the normal range.

The chemical blood findings in Table I reveal no evidence of acidosis when the CO₂-capacity values are compared with those recorded by French (1938) during *T. congolense* infection, and with the normals established for Zebu stock by Jones (1943).

There is, however, a significant increase in the level of the resting blood-glucose. Although the values all fall within normal bovine limits, reference to fig. 1 shows that the majority are above the class mode for blood-glucose and higher than the values obtained in the controls. Previous findings at this laboratory during the course of *T. congolense* infection were mostly below the class mode, excepting a few higher terminal values (Fiennes *et al.*, 1946).

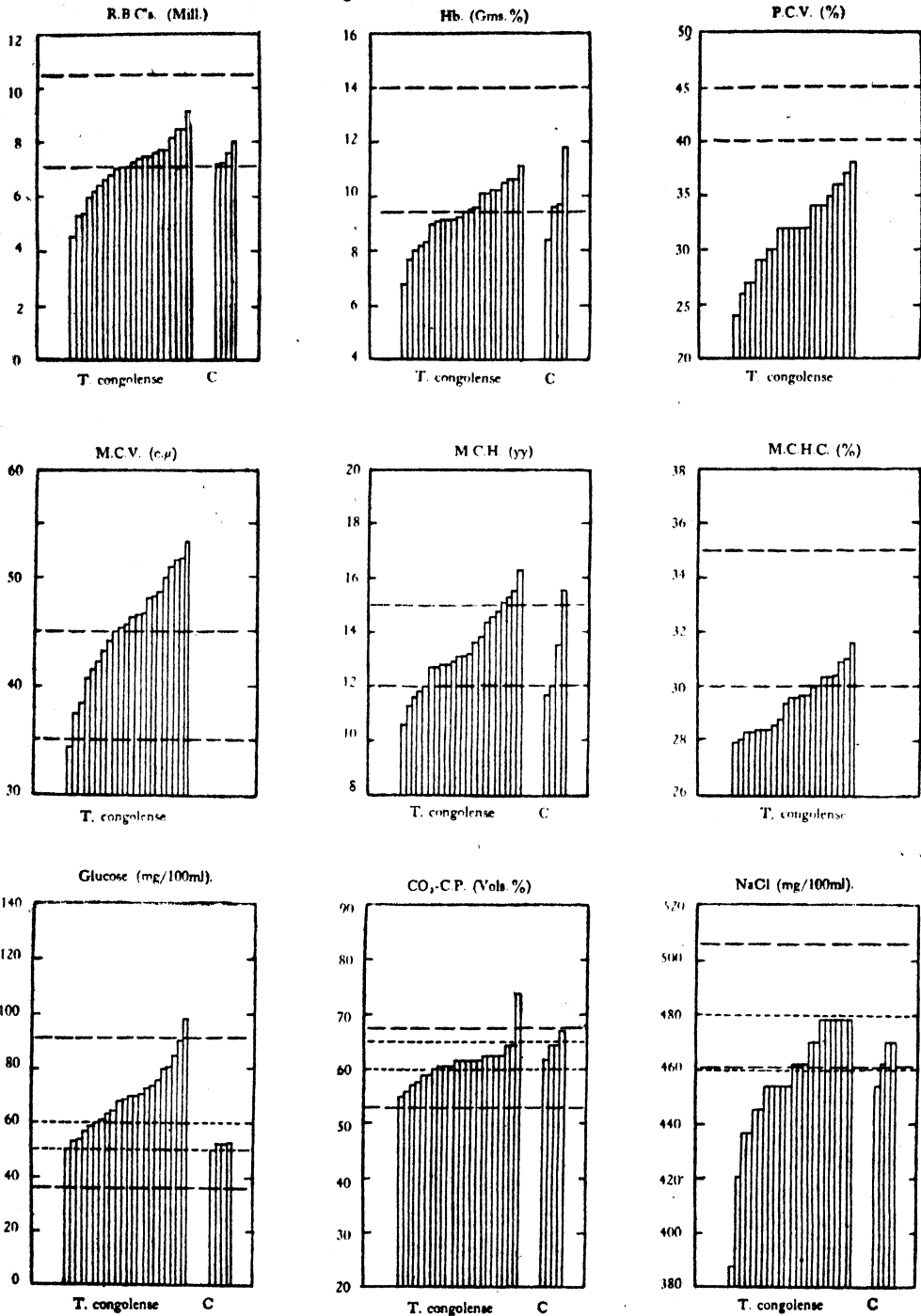


FIG. 1. Blood constituents of the 22 bovines infected with *T. congolense* and of four controls. In each case the normal range (long-dashed line) for healthy Ankole and Zebu stock, established at the Veterinary Research Laboratory, Entebbe, Uganda, is also shown, together with the class mode (short-dashed line) for the chemical constituents. C = Controls.

2. Intravenous Glucose-Tolerance Tests

The results of the intravenous glucose-tolerance tests in the 22 bovines infected with *T. congolense* and four controls are shown in Table II, together with data for the removal-rate of glucose from the blood, the glucose-tolerance or 'G' area and the hyperglycaemic index. The glucose-tolerance (G) area was determined according to the criteria of Himsworth (1939), the area measured by planimeter readings being bounded by the glucose-tolerance curve and a horizontal base level with the resting blood-sugar. Data are also included regarding the glycosuria produced by the injection, together with the values obtained for the descending renal threshold, where possible.

The blood-sugar curves obtained in the 26 intravenous tests are illustrated in fig. 2, together with the degree of glycosuria in each case. It is evident that the *T. congolense* cases produced a high type of curve compared with the controls. Though the maximal hyperglycaemia is approximately the same in both, the average values obtained being 165.2 mgm./100 ml. and 164.3 mgm./100 ml. respectively, in no other respect are the curves comparable. Whereas in the controls the blood-sugar falls rapidly to normal within two hours and remains only a comparatively short time above the renal threshold, in the infected animals the rate of fall is considerably slower, the resting level not being reached within two hours, and the blood-sugar remains above the threshold throughout the test in the majority of cases. The glycosuria, in consequence, persists for much longer than in the controls, frequently extending over the whole two-hour period of observation, and it was still present in one case which was examined five and a half hours after injection. The removal-rate of glucose from the blood is very much lower, ranging from 0.3 to 0.8 per cent. per minute, compared with the range of 1.0 to 2.8 per cent. per minute in the controls.

There is thus a considerable decrease in glucose tolerance in the infected animals, since the bovine species is independent of the influence of carbohydrate in the preceding diet, as has already been shown. The slower utilization of glucose in the infected animals compared with the controls is illustrated in fig. 3, which shows the average curve obtained in each. This decreased tolerance is also evidenced by the fact that the mean glucose-tolerance area of the *T. congolense* cases is nearly twice that of the controls (the values being 7,949 mgm./min. and 4,288 mgm./min. respectively), whilst the hyperglycaemic index extends far beyond the normal range.

The degree of impairment assessed from the intravenous curves ranges from slight impairment, in the case of bovine 5317, to gross impairment, in bovine 5329. Bovine 5317, which gave the curve most nearly approximating to the highest normal, must be considered as indicating some dysfunction, since the one-hour blood-sugar was still above the renal threshold, which was never the case in the controls, and both the glucose-tolerance area and the hyperglycaemic index are considerably above the average normal. The degree of impairment evidenced in the diseased animals has been classified, according to the data given in Table II, into the following types: eight mild, ten moderate and four severe.

No correlation could be observed between the degree of impairment and the blood physical findings, the coefficient of correlation being -0.048 for the hyperglycaemic index and mean corpuscular haemoglobin, and $+0.077$ for the hyperglycaemic index and mean corpuscular volume.

It should be recorded that none of the *T. congolense* bovines exhibited glycosuria during frequent tests made before glucose injection, despite the pronounced higher range

TABLE
Intravenous glucose-tolerance tests (0.2 gm./kgm. glucose)

No.	Weight (kgm).	Blood-sugar in mgm./100 ml.					
		Before injection	Time after completion of injection				
			2 minutes	15 minutes	30 minutes	60 minutes	120 minutes
<i>T. congolense</i> :							
5313	283	70.7	170.2	(16) 157.0	(31) 153.8	137.0	117.6
5314	239	80.6	168.8	152.6	155.6	151.4	142.8
5316	264	54.2	(6) 157.5	133.3	130.7	(61) 113.6	97.1
5317	229	57.3	151.0	(16) 128.2	119.7	103.6	79.0
5318	267	60.2	152.7	(16) 134.2	123.5	111.7	88.9
5319	205	74.3	182.6	150.4	147.6	139.9	119.7
5320	248	69.7	173.2	(17) 157.5	(32) 142.8	(62) 137.9	(124) 121.2
5322	225	69.9	149.2	(16) 137.0	122.7	117.0	(122) 102.6
5323	241	80.0	167.4	(18) 152.5	(33) 152.0	(62) 141.8	137.0
5324	265	69.4	(3) 161.2	148.1	(31) 138.9	(61) 131.6	103.1
5325	241	73.0	171.6	157.5	146.0	131.6	100.5
5327	246	63.5	(3) 153.8	(19) 135.1	(31) 129.8	117.6	103.1
5328	226	53.0	(3) 152.7	128.2	128.2	120.5	103.6
5329	228	76.3	186.8	186.8	(31) 164.0	163.2	151.0
5331	230	61.2	160.0	142.8	(31) 137.9	126.6	98.0
5332	216	59.2	151.5	129.8	134.2	119.0	101.0
5333	260	49.7	149.8	(16) 135.1	127.4	(61) 110.5	93.0
5335	204	64.3	160.0	144.9	136.0	125.0	114.9
5336	207	90.5	192.2	(16) 165.2	163.2	161.2	147.6
5337	250	67.8	(4) 175.4	153.8	148.1	(61) 133.3	101.0
5338	216	85.1	171.6	(16) 153.2	(31) 154.6	150.0	129.8
5339	204	97.6	(3) 175.4	152.0	(29) 161.7	(63) 148.1	123.5
Extreme range		49.7/97.6	149.2/192.2	128.2/186.8	119.7/164.0	103.6/163.2	79.0/151.0
Mean (22 <i>T. congolense</i>)		69.4	165.2	147.0	141.7	131.5	112.5
<i>Controls</i> :							
4988	275	51.7	156.2	112.4	70.9	35.9	55.4
5274	143	55.8	152.6	113.6	86.9	63.9	53.6
5294	130	47.9	(3) 149.2	106.4	79.7	63.1	54.3
5298	146	55.4	156.2	125.0	99.0	69.9	60.8
Extreme range†		47.9/67.6	149.2/183.4	106.4/150.4	70.9/124.2	35.9/95.7	53.6/79.7
Mean† (14 controls)		58.1	164.3	127.3	101.1	74.6	65.2

NOTES. *Hyperglycaemic index calculated on formula: $H.I. = \frac{(\text{Blood-sugar level at 2 hours}) - (\text{fasting-level})}{(\text{Maximum blood-sugar level}) - (\text{fasting-level})} \times 100$

[McCowan and Quastel (1931)].

†Extreme range and mean (14 healthy bovines) includes data compiled from results of tests (at 0.2 gm./kgm. glucose) published by Bell and Jones (1945).

Removal-rate of glucose calculated by the method of Hamilton and Stein (1942).

of their resting blood-sugar values, which extended up to the normal bovine renal threshold. Tests for the presence of acetone in the urine during the course of the glycosuria following injection were negative in the few cases examined. The descending renal threshold in the infected animals shows a slightly higher mean value and range compared with the controls,

II
in 22 bovines infected with *T. congolense* and in four controls

Removal-rate of glucose % per minute	Glucose-tolerance area (G) mgm./minute	Difference resting glucose and 2 hours glucose (mgm.) [d]	Hyper-glycaemic index* [H.I.]	Descending renal threshold mgm./100 ml. blood-sugar	Duration of glycosuria. Period after injection	Classification of impairment (based on [d] and [H.I.])
0.3	8,670	46.9	47.3	— — —	90 minutes (+)	Moderate
0.3	8,640	62.2	70.5	142—	122 "	Severe
0.8	8,010	42.9	41.7	113-118	50 "	Moderate
0.8	5,940	21.7	23.1	107—	42 " (+)	Mild
0.7	6,420	28.7	31.0	116-123	32 "	"
0.7	8,250	45.4	42.0	— — —	125 " (+)	Moderate
0.7	9,090	51.5	49.5	121-125	114 "	"
0.7	6,540	32.7	41.5	103—	133 "	Mild
0.3	8,190	57.0	65.5	— — —	127 " (+)	Severe
0.6	7,350	33.7	36.7	133-135	41 "	Mild
0.6	7,320	27.5	27.7	101—	123 " (+)	"
0.7	7,230	39.6	44.2	111-113	74 "	Moderate
0.6	8,700	50.6	50.6	115-120	58 "	"
0.5	11,010	74.7	68.0	— — —	To 5½ hours	Severe
0.5	8,190	36.8	37.3	— — —	119 minutes (+)	Mild
0.4	7,860	41.8	45.5	116-120	58 "	Moderate
0.6	8,160	43.3	43.3	103-108	72 "	"
0.6	8,310	50.6	53.0	115—	122 "	"
0.6	8,670	57.1	56.0	— — —	125 " (+)	Severe
0.6	8,160	33.2	30.7	— — —	121 " (+)	Mild
0.3	8,070	44.7	51.5	— — —	155 " (+)	Moderate
0.3	6,090	25.9	33.3	— — —	122 " (+)	Mild
0.3/0.8	5,940/11,010	21.7/74.7	23.1/70.5	101/142	32 minutes/ 5½ hours (+)	—
0.6	7,949	43.1	45.0	118.6	—	—
2.8	2,130	3.7	3.5	65—	34 minutes	Nil
2.0	3,180	—2.2	1.0	113—	16 "	"
2.3	3,990	6.4	6.3	— — —	7 " (+)	"
1.6	3,990	5.4	5.3	— — —	23 " (+)	"
1.0/2.8	2,130/5,850	—2.2/16.4	0.9/15.3	65/130	16/67 minutes	Nil
1.8	4,288	7.1	6.9	99.5	—	"

Descending renal threshold calculated from curves in fig. 2, and taken as the blood-sugar level corresponding to glycosuria = + (Benedict's qualitative test).

Duration of glycosuria taken as the maximum period after injection giving glycosuria = + (Benedict's qualitative test).

Glucose-tolerance area (G) calculated from areas of curves in fig. 2 measured by planimeter readings.

DISCUSSION

French (1938) records a death-rate in *T. congolense* infection of bovines of 33 per cent., with survival of his remaining animals up to 44 weeks, while Fiennes (1945) states, on the other hand, that a tendency towards a 100 per cent. death-rate is to be expected in this disease. Our results show a 16 per cent. death-rate, the deaths occurring within three months of infection, the remainder surviving one year after infection.

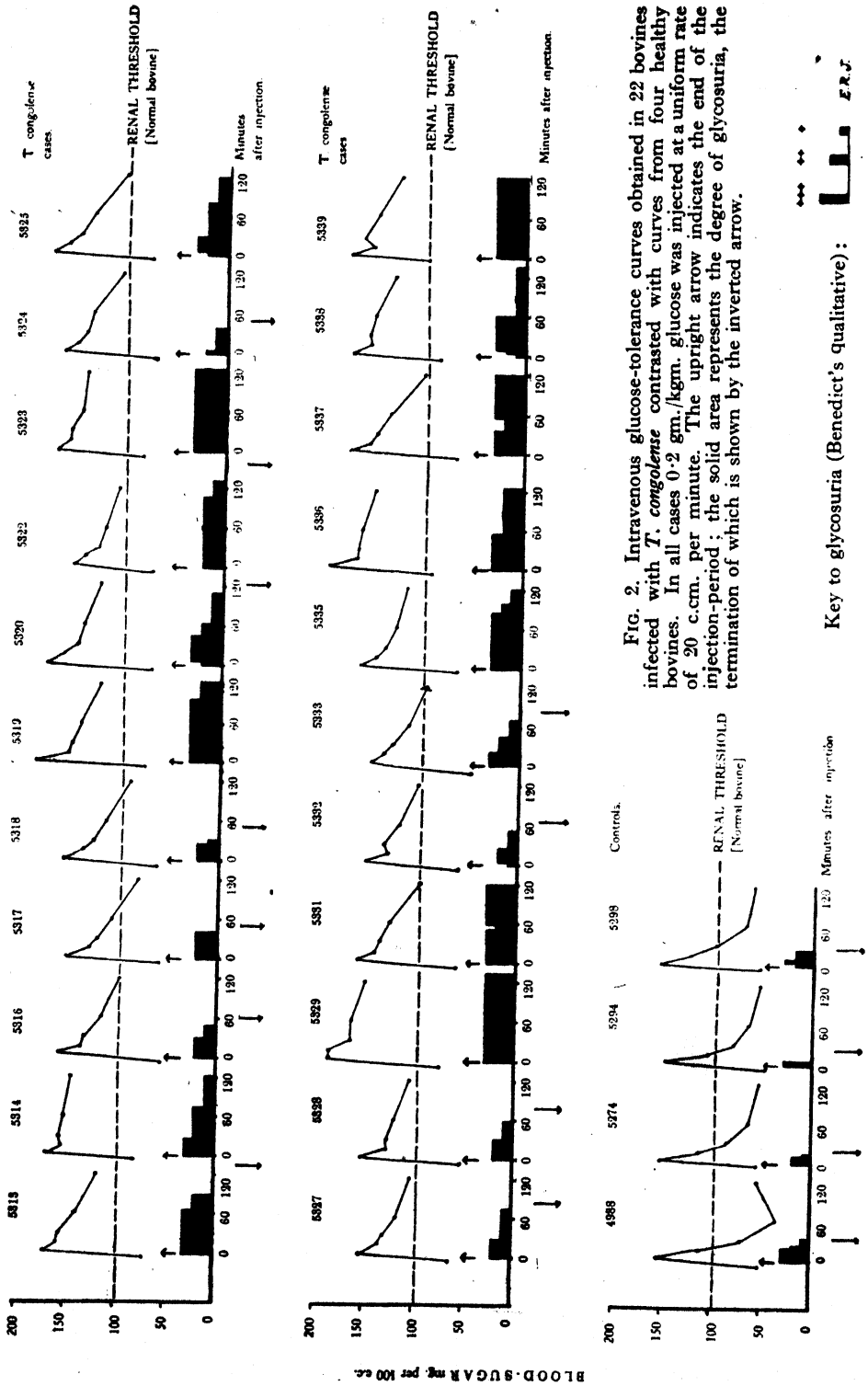


FIG. 2. Intravenous glucose-tolerance curves obtained in 22 bovines infected with *T. congolense* contrasted with curves from four healthy bovines. In all cases 0.2 gm./kgm. glucose was injected at a uniform rate of 20 c.cm. per minute. The upright arrow indicates the end of the infection-period; the solid area represents the degree of glycosuria, the termination of which is shown by the inverted arrow.

Examination of our experimental animals seven months after infection, when they had passed into the chronic stages of the disease, showed no clinical abnormality. Weight lost during the crisis-period had been recovered, although most of the animals were still showing scanty *T. congolense* in the peripheral blood. The animals had apparently become premunized. The blood picture at the seven-month period after infection shows a varying degree of recovery from the marked anaemia associated with crisis in *T. congolense* infection.

The finding of a slight degree of hyperglycaemia in the pre-mortal stage of *T. congolense* infection, following the hypoglycaemia generally recorded at the time of crisis, is in agreement with the results recorded by Fiennes *et al.* (1946). Conclusive evidence of decreased glucose tolerance in trypanosomiasis does not appear to have been recorded

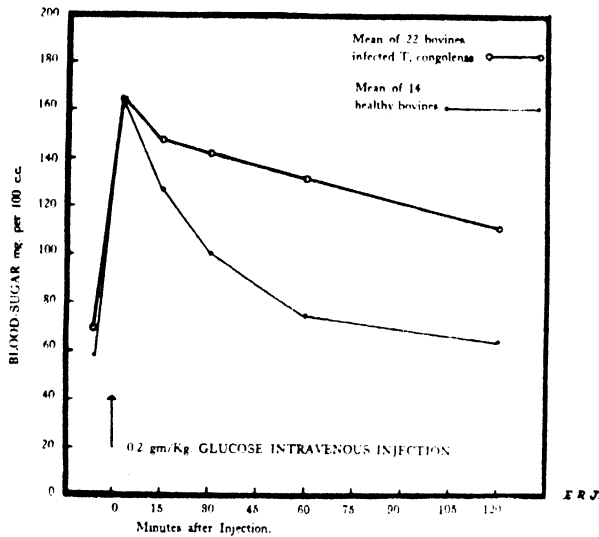


FIG. 3. Intravenous blood-sugar curves in *T. congolense* infected cattle contrasted with normals.

previously. Wormald (1932) concluded from oral glucose-tolerance tests in man that there is no serious impairment of the capacity of the liver to deal with glucose, although 'several of the glucose tolerance curves were of the lag type.' Examination of the curves by means of the calculated hyperglycaemic index from some of his infected subjects, however, shows values of 37-88, compared with the highest normal value of 25. The low oral curves in several of his infected cases and controls were probably due to the high carbohydrate diet of the Africans concerned, the influence of which has already been demonstrated by Himsworth (1939).

The disturbance of carbohydrate metabolism in chronic trypanosomiasis bears a similarity to the hyperglycaemic phase of alloxan diabetes in rabbits (Duffy, 1945). A lesion in the islets of Langerhahn in *T. congolense* cases similar to that produced in rabbits by alloxan has already been described by Fiennes *et al.* (1946). A detailed description of the lesions produced in our cases of *T. congolense* will be published by one of us (F. R. B.) at a later date.

The slightly raised renal threshold found in the chronic stage of *T. congolense* infection is at variance with the hypothesis of Fiennes *et al.* (1946) that the severe kidney lesions reported by them might lower the threshold. Until evidence is available as to the level of

the threshold in the acute stage of the disease, our findings cannot be taken as disproving this hypothesis, since a low threshold may possibly exist in the hypoglycaemia phase of trypanosomiasis.

SUMMARY

1. In a group of 26 adult Zebu cattle infected with *Trypanosoma congolense* only four deaths occurred, the remainder surviving beyond one year.

2. Studies made on 22 bovines in the chronic stage of the disease, seven months after infection, showed no abnormality clinically, weight-loss almost restored, and blood physical findings indicating a varying degree of recovery from the anaemic crisis, although most of them still showed trypanosomes in the blood.

3. No evidence of acidosis was apparent, but there was a significant increase in the level of the resting blood-sugar, constituting a slight degree of hyperglycaemia, and subnormal blood-chloride values.

4. Intravenous glucose-tolerance tests produced a high type of curve, with prolonged glycosuria and failure to return to normal within two hours, thus indicating a considerable decrease in glucose tolerance.

5. The impairment shown by the tests could be classified into eight mild, ten moderate and four severe types, showing a hyperglycaemic index ranging from 23 to 70 and a glucose-tolerance area approximately twice that of the controls.

6. The decreased carbohydrate tolerance and the slight elevation of the blood-sugar value is compared with the hyperglycaemic phase of alloxan diabetes.

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AN APPARATUS FOR COLLECTING BLOOD-SUCKING MITES

BY

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During a recent investigation of the life-cycle and habits of the blood-sucking mite *Liponyssus bacoti*, and of the part played by it in the transmission of the filaria worm *Litomosoides carinii* to the cotton rat *Sigmodon hispidus* (Bertram *et al.*, 1946), it was frequently necessary to isolate the mites from the sawdust or mixed sand and earth used as the bedding-material for the rats. This proved a difficult and time-consuming operation, and the apparatus about to be described was designed to facilitate the work.

The principle of the apparatus is that of a heated cylinder embracing and moving slowly along a long glass tube containing the infested material; the cylinder in its progress drives the mites before it into a terminal chamber.

Mite-infested cotton fibre is not suitable for direct treatment with the apparatus, as the mites become trapped in the fibres and are destroyed. This difficulty has been overcome by teasing out such materials into sawdust, which is then treated as described below.

The apparatus has been extremely useful in saving the time and the labour involved in isolating mites from bedding and transferring them to small containers.

Although the device has been used only for the type of materials mentioned, it is thought that the present note may be of interest to other workers concerned with the separation of small arthropods from particulate material. Some preliminary observations have been made on the effect of contact insecticides upon the mites, using the apparatus as a means of inducing the mites to move over a known distance of treated surface in a known period of time. The apparatus may have some general applications in this type of work.

THE APPARATUS

FIG. 1 (A), (B) AND (C)

Preliminary trials of the principle involved were made using a sheet-tin ring 1 in. broad heated by a spirit-lamp in the manner employed for a microscope warm-stage, the ring and the lamp being mounted on a platform which was moved by hand. To allow some control over the heat generated by the ring and to save time and labour, an electrically heated cylinder and a method of mechanical traction were devised.

The apparatus in its present form consists of five main parts:

1. A sample-tube to hold the material to be treated.
2. A framework to support the sample-tube in a fixed position.
3. A heating unit mounted on a trolley.
4. A traction system to move the trolley.
5. A collecting-chamber incorporating a device for controlling the condensation of moisture within the sample-tube.

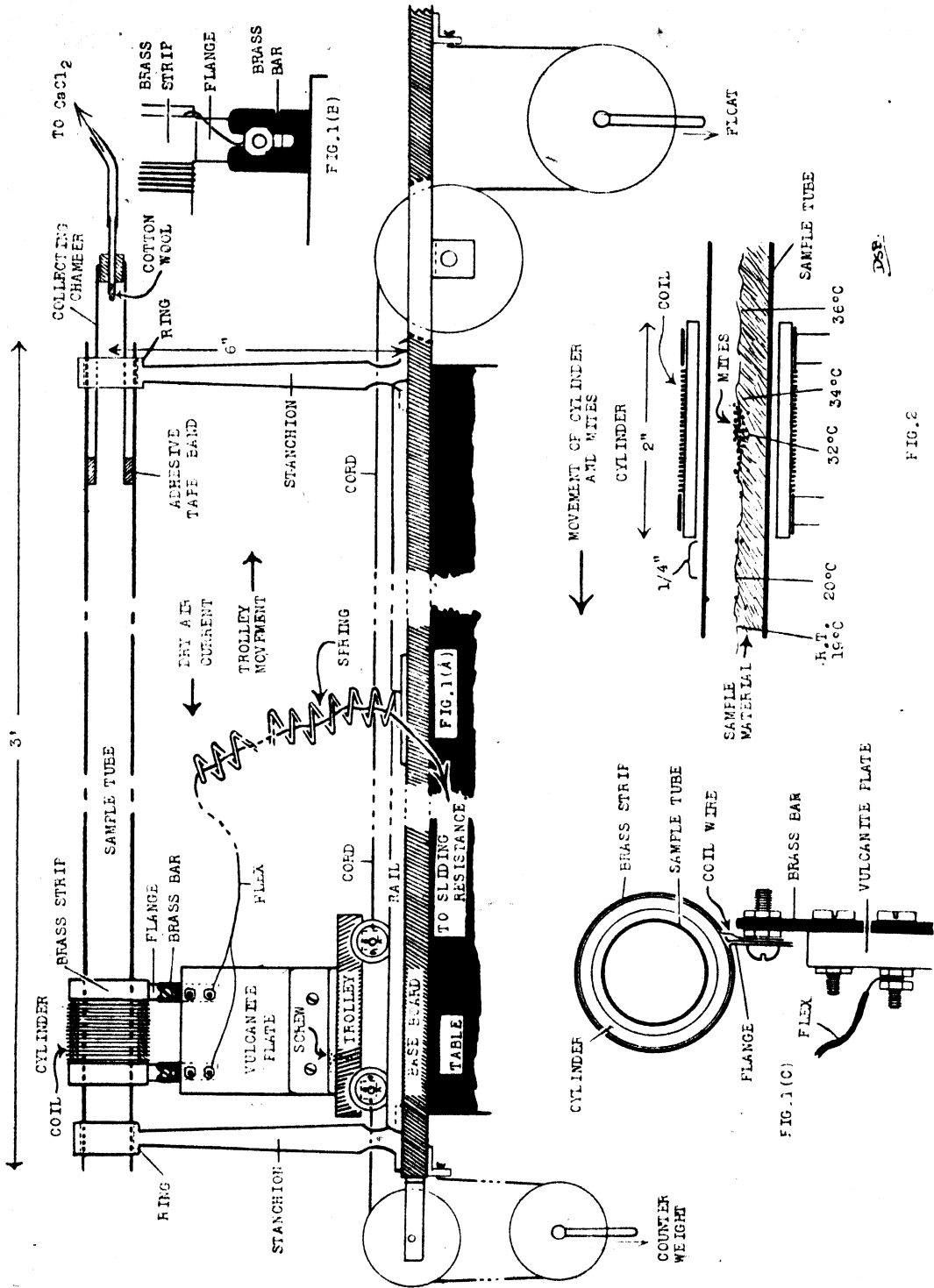


FIG. 1. (A)—Lateral view of the apparatus. (B)—Detail of the attachment of the cylinder to the trolley. (C)—Transverse sectional view of the cylinder, etc.

FIG. 2.—Longitudinal sectional view of the cylinder, etc., to show the critical zone for the mites.

1. *The Sample-Tube* is made of glass 1 mm. thick and is 3 ft. long with an outside diameter of 1 in.

2. *The Framework* consists of a wooden base-board 3 ft. 11 in. by 1 ft. by $\frac{3}{4}$ in. thick. Two brass stanchions are fixed to this board, one stanchion at one end of the board and the other in line with it at a distance of 2 ft. 11 in. Each stanchion is in the form of a ring at the top, and the sample-tube rests in the rings at a height of 6 in. above the board. A hole through the base of each stanchion, a slot in the base-board, and four L-brackets screwed to the board, as shown in the diagrams, are required to accommodate the traction system. A $3\frac{1}{2}$ in. gauge brass rail-track runs between the stanchions. The base-board serves as a platform to hold parts of the electrical equipment and the device for controlling condensation. The board rests on a table which is of a suitable size and height to permit of free movement of the terminal pulley systems [fig. 1 (A)].

3. *The Heating Unit and Trolley*. The heating unit is a resistance-coil of 48 ohms made of 62 turns of nickel silver resistance-wire (resistance of 0.5 ohm per foot) wound round the outer surface of a fire-clay cylinder 2 in. long with an external diameter of $1\frac{1}{2}$ in. and a wall thickness of $\frac{1}{8}$ in. Round each end of the cylinder there is a thin brass strip, the ends of which are turned away from the cylinder and, held together firmly by a long bolt and nut, form a flange for attaching the cylinder to the trolley [fig. 1 (C)]. The wiring of the coil passes under the brass strip at each end of the cylinder and is secured between the flange and the nut.

The trolley runs on four flanged wheels retained on fixed axles by split pins, the axles being screwed to the underside of the trolley platform. The platform is of wood 4 in. by 3 in. by $\frac{3}{4}$ in. thick, and carries medially a vulcanite plate $3\frac{1}{2}$ in. high by $2\frac{1}{2}$ in. by $\frac{1}{2}$ in., which is held on the platform by a single screw passing through an L-bracket bolted to the vulcanite plate. Two brass bars are doubly bolted to this plate and they extend above its upper edge by about $\frac{3}{4}$ in. The bars are sited to correspond with the position of the bolted flanges on the cylinder, and the long bolt of the flanges slips into a slot in the upper part of the brass bars. A second nut on each bolt secures the cylinder to the trolley [fig. 1 (B) and (C)].

This type of attachment between the trolley and the cylinder enables the operator to make adjustments in the horizontal setting of the cylinder, and lateral adjustments are made by swivelling the vulcanite plate on the screw which holds it to the trolley platform. In principle the cylinder should require to be set only once in true alignment with the sample-tube, but in practice minor variations in the shape of different sample-tubes make some provision for adjustments necessary.

The cylinder is set to pass freely along the sample-tube with an all-round clearance of approximately $1/10$ in.

The heating coil is connected to a 5 amp. electric point on the 230 volt A.C. supply from the mains, but, in order to give some control over the heat generated, a sliding resistance is included in the circuit and is mounted on the base-board. The flex from the sliding resistance is passed up through a flexible brass spring, 9 in. high and $\frac{3}{4}$ in. in diameter, screwed to the base-board at a point about half-way along and to one side of the trolley track. This arrangement eliminates the possibility of the flex obstructing the movement of the trolley. The flex leads to the lower of the two bolts fixing each brass bar to the vulcanite plate, and by engaging a wire to each of the bolts by means of a second nut the circuit is completed.

The heating cylinder and the trolley weigh 430 gm.

4. *The Traction System.* The trolley is drawn along the track by means of a cord tied to the front axle. The cord passes through the base of the stanchion at the end of the track and over a 3 in. pulley which is held in the slot of the base-board by an axle mounted on an L-bracket beneath the board. From this pulley the cord passes down and round under a second pulley and is tied finally to an L-bracket under the base-board. A wooden float, 9 in. in diameter and weighing 820 gm., is hooked on to the second pulley. The water to operate the float is contained in a five-gallon drum 18 in. in height with a tap at the lower limit. A suitable speed for the trolley is obtained by controlling the rate of flow of the water from the drum.

The cord attached to the rear axle passes through the base of the other stanchion and over a 1 in. pulley carried on an L-bracket on the end of the base-board. A second pulley to which is attached a 350 gm. counter-weight is substituted for the pulley and float at the other end of the system, but otherwise the arrangement is similar at both ends.

5. *The Collecting-Chamber and Condensation Control.* The collecting-chamber consists of glass tubing about 3 in. long and $\frac{1}{2}$ in. wide, one end of which is closed by a rubber stopper penetrated by a piece of narrow-bore tubing plugged firmly with cotton wool at its inner end. Adhesive tape is wound round the other end of the 3 in. tube to form a band thick enough to allow the collecting-chamber to slide into the wider sample-tube and yet give a close fit between the sample-tube and the tape. A rubber stopper or a firmly packed plug of cotton wool is used to seal the collecting-chamber after its removal from the apparatus.

Even with comparatively dry material condensation will occur in front of the cylinder, on the inner surface of the sample-tube, during the traverse of the heating coil. The extent to which this develops depends on the room-temperature, the temperature of the glass, and the moisture content of the sample, and it results in a variable number of mites being immobilized in the droplets and ultimately killed by the heat. This difficulty has been overcome by pumping air, which has been passed over calcium chloride, into the sample-tube in the reverse direction to that of the movement of the trolley. The dried air pumped by a 'Marco' aquarium-aerator pump enters the sample-tube through the fine-bore tube in the stopper of the collecting-chamber.

By this method condensation of moisture is restricted to a zone 2 in. or more behind the coil and does not interfere with the movement of the mites. The delivery of dried air from the other end of the sample-tube was not successful.

THE METHOD OF USING THE APPARATUS

The sample-tube is removed from the rings and closed by a plug of cotton wool inserted for about 2 in. into the end to which the collecting-chamber will be attached. The tube is now held vertically with the plug at the lower end, and the material to be treated, up to a maximum of 20 gm. of sawdust, is added through a filter-funnel. The open end of the tube is then sealed with a cotton-wool plug, which is pushed down the tube for 3 in. or more according to the amount of material which has been added; in general, 6 in. is a suitable distance. The tube is next held horizontally and tilted up and down until the sawdust is evenly spread between the plugs and occupies approximately the lower half of the tube. Retaining the tube in a horizontal position, it is slipped into the ring of one stanchion, through the heating cylinder, to the other

stanchion. A strip of adhesive tape is applied to fix the tube to the stanchion from which the trolley starts. At the other end the wool plug is removed and replaced by a collecting-chamber and its attachment for the supply of dried air. It is important to ensure that the layer of sawdust comes right up to the entrance of the collecting-chamber, as an exposed hot glass surface at this point will kill mites which fall from the entrance to the chamber in the final phase of the isolation. The adhesive tape prevents the chamber from heating up too rapidly.

The drum is now filled with water to a height which will bring the heating cylinder to the level of the remaining cotton-wool plug, and before setting the trolley in motion the coil should be allowed to heat up for about five minutes. The current of dried air should be pumped in immediately and be continuous throughout the operation.

The temperature generated in the cylinder depends upon the position selected on the sliding resistance. In practice the author has operated the apparatus with a temperature of 90° C. on the internal surface of the fire-clay cylinder. During four hours of continuous heating at a room-temperature of 18–20° C. there was little fluctuation in the temperature of the cylinder.

The trolley moves in a series of jerks of about 1/10 in. or less, and speeds of about $\frac{1}{2}$ in. in one minute, or less, have been found suitable for driving the mites without any significant loss.

The cylinder may be started at any point along the tube to suit the size of the sample, and the time taken to complete an operation varies accordingly. In general, one to two hours is sufficient for one isolation, during which time the operator need attend the apparatus only at the beginning and at the end of the traverse. It is important that the operator supervises the end of the movement, as condensation of moisture in, and over-heating of, the collecting-chamber is liable to develop after the cylinder has overlapped the band of adhesive tape.

AN EXAMPLE

FIG. 2

In one routine treatment of 20 gm. of sawdust containing mites teased out from cotton fibre, a thermometer was laid on the surface of the sawdust, with the mercury bulb about 10 in. from the centre of the heating cylinder when it was at the starting-point.

The movement of the cylinder, timed over three sections of three inches, gave an average speed of 0.36 in. in one minute, and the total length of the traverse was 25 in.

The apparatus was operated with the usual temperature of 90° C. on the inner surface of the cylinder, and the room-temperature was 19° C. The test-thermometer registered 19° C. until its tip was $\frac{1}{4}$ in. from the leading edge of the cylinder, but thereafter the temperature rose rapidly, reaching 30° C. with the bulb nearly half-way through, and 36° C. when the bulb was just showing behind, the cylinder. A maximum temperature of 44° C. occurred with the bulb about $1\frac{1}{2}$ in. behind the cylinder, and, by the end of the operation, the reading was 23° C.

Numerous mites of different stages and moving in various directions occurred for several inches in front of the cylinder, but the critical level for directing the movement of the mites was slightly behind the mid-point of the cylinder, where the sawdust was judged to have a temperature of between 32° C. and 34° C. Throughout the traverse, mites were overtaken by the cylinder, and as the operation continued this zone of agitated

mites became progressively more apparent. This operation yielded 113 mites, comprising about 60 gorged and unfed females, the remainder being males and nymphs in varying degrees of engorgement or unfed.

The apparatus has so far been used successfully for isolating such active stages of the mite as may occur in random samples of material. Critical tests on the percentage loss of different stages have not been undertaken. There is no apparent loss of gorged and unfed females or of males. Gorged and unfed first nymphs have been recovered from the collecting-chamber and in some cases a few second nymphs have been observed. The losses in the case of the relatively inactive second nymph are likely to be considerable in any given instance. The relatively inactive larva has never been isolated, and it is probable that eggs, if present, are to a large extent destroyed.

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PROLONGED ORAL ADMINISTRATION OF MEPACRINE

III.—THE CLINICAL EFFECTS IN WOMEN

BY

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In an investigation of the clinical effects in women of a long-continued course of mepacrine 109 women undergraduates from the University of Oxford were kept under observation for periods up to 12 months. Attention was paid particularly to the following points :

1. The possible occurrence of toxic symptoms.
2. The general health of the volunteer, as assessed by clinical observation, weight, pulse-rate and blood picture.
3. The effects, if any, on menstruation.
4. The distribution of mepacrine in the skin and hair.
5. The mepacrine concentration obtaining in the blood.

DESCRIPTION OF EXPERIMENTS

The volunteers were divided at first into five groups and put on the following dosage régimes :

- (a) Mepacrine 0.1 gm. before breakfast every day (0.6 gm. weekly).
- (b) Mepacrine 0.1 gm. before breakfast on Monday, Tuesday, Wednesday and Thursday (0.4 gm. weekly).
- (c) Mepacrine 0.2 gm. before breakfast on Monday and Thursday (0.4 gm. weekly).
- (d) Mepacrine 0.05 gm. before breakfast on week-days and 0.1 gm. on Sundays (0.4 gm. weekly).
- (e) A control-group on a placebo, prepared by Imperial Chemical (Pharmaceuticals) Limited, indistinguishable in size, taste and appearance from the mepacrine tablet used.

This control-group was divided and attached to the previous four groups. Their tablets were taken according to their group-régime and they were unaware that they were taking the placebo.

After three months all volunteers were put on mepacrine 0.1 gm. before breakfast daily (0.7 gm. weekly), and the controls were put on a similar régime ; this dosage was maintained for the rest of the experiment.

SYMPTOMS

Volunteers were seen at intervals of approximately two months throughout the experiment. All symptoms reported by each volunteer were noted, but particular care was

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taken to find out whether any of the following symptoms were complained of: vomiting, loose stools, dyspepsia, flatulence, abdominal pain, hepatic pain, sleeplessness, nightmare, loss of concentration, headaches, palpitations, muscular aching and falling out of hair.

In the control-group there were no symptoms of any importance, except in the case of one subject who complained of headache throughout the experiment; this ceased after she stopped taking the placebo.

With the exception of one volunteer, who was subsequently found to have colitis, none of the subjects taking mepacrine complained of severe symptoms. Twelve volunteers complained of symptoms which were considered to be wholly or partly due to mepacrine. These symptoms were all gastro-intestinal—mostly loose stools—and tended to occur early in the course and to disappear spontaneously despite the continuance of mepacrine. Vomiting occurred only in those on mepacrine 0.2 gm. twice weekly (three out of a group of 20 volunteers). That the symptoms were physical and not functional was shown by their disappearance when the placebo was substituted for mepacrine without the volunteers' knowledge. Radiological examination of the gastro-intestinal tract in these cases showed no abnormality, and no significant changes occurred in the faecal flora or the skin-patch test, where 1 per cent. mepacrine ointment gave uniformly negative results. There were no gastro-intestinal symptoms in the control-group.

Three volunteers developed psychotic disturbances:

Volunteer No. 127. Started on mepacrine 0.6 gm. weekly, increasing to 0.7 gm. weekly after two months. In her third month she complained of thirst, frequency (urine was normal) and 2-3 loose stools daily. These symptoms continued for the next three months, improving steadily without treatment. X-ray of the gastro-intestinal tract showed no abnormality. At the end of her sixth month she was given a provocative dose of mepacrine 1.0 gm. followed by a barium meal (Army Malaria Research Unit and Nuffield Institute for Medical Research, 1946), after which her experiment was ended and no further mepacrine was given. Twenty-five days later she became stuporose and was taken to hospital, where she was later diagnosed as suffering from 'schizophrenic disorder of an episodic type.' This psychosis was considered to have no connection with mepacrine administration.

Volunteer No. 146. Started on mepacrine 0.6 gm. weekly, increasing to 0.7 gm. weekly after two months. She complained of no symptoms, but after five and a half months she developed hypomania and was admitted to hospital two days later in a confused state; she vomited once a day for the first nine days. There was no previous history of psychotic disturbance. She recovered completely in six weeks. Opinion of psychiatrists was divided on whether mepacrine could have contributed to the precipitation of her psychosis.

Volunteer No. 162. This volunteer was placed on mepacrine 0.05 gm. on weekdays and 0.1 gm. on Sundays (0.4 gm. weekly). She developed a confusional psychosis after the fourth dose, i.e., after a total quantity of 0.2 gm. mepacrine. Mepacrine was discontinued after 0.5 gm. in all had been given. There was a previous history of psychological instability. There appeared to be no connection between such a small quantity of drug and the development of this psychosis.

GENERAL HEALTH

Volunteers were examined every two weeks. No clinical evidence of deterioration of health was found. No significant changes in weight were observed in either mepacrine- or control-group after six months. No significant changes occurred in the red-cell count, the white-cell count, the haematocrit or the erythrocyte sedimentation-rate (Army Malaria Research Unit, 1945).

MENSTRUATION

A calendar chart was kept for each of 83 volunteers, on which was marked the dates over which each period extended. A record was made of the normal intermenstrual interval and the duration of the period prior to the commencement of the mepacrine course. Subsequently the volunteer was asked whether there had been any alterations in the amount of loss or the degree of pain, and whether any other menstrual changes had been observed.

The results were as follows :

Duration of period. In four subjects the menstrual period was shortened by one day, in five lengthened by one day after the commencement of the mepacrine dosage. One control-subject observed an increase of one day.

Menstrual loss. Thirteen volunteers reported a reduction in loss during the experiment, four reported an increase. One reported a one-day intermenstrual loss occurring twice during the experiment. One control-subject reported a decrease in menstrual loss and two an increase.

Menstrual pain. Seven volunteers reported a diminution of menstrual pain; two reported an increase. No alteration was reported by the controls.

Intermenstrual interval. Two volunteers showed an increase in intermenstrual interval and three a decrease. In four the interval was more irregular on mepacrine, and in four more regular. One showed four months' amenorrhoea. In the controls one showed a decrease in the interval and one was more irregular. In no case was the disturbance more than trivial. These minor changes occurred in the same proportion in both the mepacrine- and the control-groups, and may therefore be regarded as normal variations.

DISTRIBUTION OF MEPACRINE IN SKIN AND HAIR

Skin. Staining occurred primarily in the pigmented areas of the skin, particularly in those exposed to direct sunlight. This was most striking in the freckled blonde subject, in whom the freckles assumed a lemon-yellow tint before the intervening skin became appreciably stained.

The staining became completely masked by sunburn in about half the number of cases. Some subjects stated that they tanned with greater speed and intensity than ever before. In the winter months, as the tan faded, typical mepacrine yellow staining reappeared.

Appreciable yellowing of the conjunctiva was never observed. (Slight staining was occasionally seen in male volunteers.)

Hair. The mepacrine content of the proximal and distal ends of the scalp hair was estimated in seven volunteers. The mean concentration in the proximal portion of blonde hair was 11 mgm./litre and of brunette 57 mgm./litre (Army Malaria Research Unit, 1946).

There was no evidence that mepacrine caused loss of hair.

PLASMA MEPACRINE CONCENTRATION

Blood was taken at intervals from volunteers 24 hours after receiving a dose of mepacrine.

The results are as follows :

TABLE

Month in 1944	Weekly dosage (mgm.)	No. of observations	Mean plasma mepacrine concentration	Standard error of mean
January	0.6	25	25	± 2.2
March	0.7	24	31	± 2.4
April	0.7	27	35	± 2.0
June	0.7	23	25	± 1.6
October	0.7	9	25	± 3.2

SUMMARY

1. Mepacrine 0.1 gm. daily was administered to women over a period of up to 12 months without producing symptoms of any consequence. Such symptoms as did occur were gastro-intestinal, and the disturbance was small.

2. Frequent clinical examinations, including weight, pulse-rates, and blood picture, produced no evidence of any effect on general health.

3. There were no gross disturbances of menstruation. Such slight variations as did occur were also noted by the controls, and may be regarded as normal for women of this age-group and occupation.

4. Yellowing occurred primarily in the pigmented skin areas and was largely or totally masked by sunburn; brunette hair contained five times as much mepacrine as blonde. No falling-out of the hair was reported.

5. A mepacrine plasma level of about 25 mgm./litre was maintained on 0.1 gm. daily.

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THE MAINTENANCE OF A COLONY OF *PHLEBOTOMUS PAPATASII* IN GREAT BRITAIN

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INTRODUCTION

The maintenance of strains of various arthropod vectors of disease is now recognized as an essential part of the laboratory routine of any institute concerned with teaching or research in tropical medicine, and at the present time many strains of tropical and subtropical arthropods are being successfully kept in this country. So far, however, no record of the similar maintenance of *Phlebotomus* has appeared in the literature. In the tropics the rearing of sandflies under artificial conditions in the laboratory has been the subject of numerous investigations, the flies being in the majority of instances maintained in connection with research programmes on various aspects of sandfly-fever, bartonellosis and leishmaniasis in man. Even in those countries, however, in which the flies occur annually in large numbers, the task of rearing them in the laboratory has been found to be by no means a simple one, and it was not until 1922 that a technique was devised whereby the flies could be maintained and their life-cycle studied under artificial conditions.

Before reviewing the literature relating to the methods used for breeding sandflies, it may be appropriate to say something of the early searches made for the breeding-places of the flies, since these yielded important information on the type of habitat frequented by the adults and on the type of food-material necessary for larval development. Early workers, such as Grassi (1907), Marett (1910), Newstead (1911) and King (1914), despite unremitting search, were able to find only a few larvae and pupae, but later workers, such as Young *et al.* (1926), appear to have achieved considerable success by the adoption of special techniques, such as the selective sieving of the larger, and the flotation of the smaller, larvae in fluid of high specific gravity. Young *et al.* summarize the results of their researches as follows: 'The breeding grounds of *P. papatasii* are in the depths of collections of slightly moist organic matter lying in hollows and depressions in broken ground and nullahs. Associated with such suitable material must be a crack or crevice by which the sandfly can obtain access to the shelter, darkness, moisture and organic matter required for breeding.'

A succession of workers have confirmed the fact that these are broadly the type of conditions required for breeding, although the breeding-areas may differ somewhat according to local topography. Marett and Newstead found breeding-places in the cracks and crevices of walls, and Wanson (1942) recovered sandflies from rather similar situations.

It is well known that sandflies are often recovered from rodent burrows, and since a proportion of those found show signs of having recently fed it is assumed that the rodents in the burrows have supplied the blood-meal. Kirk and Lewis (1940), after reviewing the relevant literature, state that in the Sudan sandflies may be captured readily from the burrows of gerbilles, aardvarks, foxes and ground-squirrels. That such sites may also form breeding-places for the larvae has recently been noted by Schourenkova (1941) and by Latyschew and Kriukowa (1941), who showed that the burrows of ground-squirrels and gerbilles are important breeding-places of the flies—an association which has an important bearing on the technique to be described later in this paper.

As regards the rearing of sandflies under artificial conditions, the principal difficulties of the earlier, as well as of the later, workers have been to establish conditions favourable to oviposition and to the development of the young larva during the period immediately following its emergence from the egg. Although Grassi and Marett appear to have been successful in inducing the gravid female fly to oviposit, it was Newstead who first described the characteristic manner in which the eggs were laid, and the extreme exhaustion of the fly brought about by the act—an observation confirmed by later workers, who noted that the majority of females died after the first oviposition. That the flies were very susceptible to dryness, although prone to be caught up in even slight films of water, was appreciated at an early stage, and Waterson (1922) was the first to develop the method of confining them in damp earthenware pots—a method which served the dual and very essential purpose of providing the degree of humidity necessary to their survival whilst eliminating the risk which ensued from the condensation of water-vapour on the inside of the vessels. By the adoption of this technique Waterson was able not only to induce the females to oviposit, but subsequently to rear the larvae beyond the second stage in their development, and he might well have succeeded in completing the life-cycle of the fly had not a series of unfortunate accidents resulted in the loss of the strain.

Whittingham and Rook (1922) were the first to establish a colony of the flies, which they succeeded in maintaining through at least three generations in Malta. Later, they were successful in rearing a brood of sandflies in this country from eggs brought over from Malta, but they apparently failed to maintain the colony. These authors employed a special chamber for the flies, consisting of a wooden box, lined on the inside with muslin and provided with a glass front and netting sleeves at each end. Local conditions favourable to oviposition and larval development were provided by means of small mounds of earth and stones, together with the faeces of lizards, crushed insects and dried human blood kept continually in a moist state. The cages were maintained at a temperature of 80° F. No mention is made of the source of the blood-meals which the flies were offered, but the construction of the box suggests that human volunteers were employed.

From 1922 onwards many different techniques have been used by various workers. All are modifications to a greater or lesser degree of the technique described by Smith (1925) and later employed by Young *et al.* and by Napier and Smith (1926). Smith used naturally fed female flies, which he confined in small glass cylinders standing in Petri dishes containing water and covered at the upper end with muslin. After two to three days the females were transferred to another glass cylinder, used as an egg-laying chamber, the floor of which was formed of a plaster of Paris tray containing pebbles, together with rabbit faeces as food for the larvae. The plaster tray rested on damp cotton wool in an earthenware pot, the requisite degree of moisture being attained by standing the pot in

water in a Petri dish. After the eggs had hatched the glass cylinder was replaced by an earthenware cover, to establish the degree of moisture and darkness necessary for larval development. Christophers *et al.* (1926) found that the plaster blocks recommended by Smith gave unsatisfactory results, and substituted Petri dishes lined with damp filter-paper, upon which the food for the larvae was spread. The chambers for the flies consisted of lamp-glasses, the space between the glass and the sides of the Petri dish being packed with damp cotton wool. The larvae were maintained in the Petri dishes until they had pupated, adults being removed as they emerged. Shortt *et al.* (1926) substituted earthenware saucers for the Petri dishes and added a small quantity of dried blood to the rabbit or goat faeces used for the larval food. Ashner (1927), however, found that this food-material gave unsatisfactory results, as it tended to propagate moulds which inhibited the normal development of the young larvae. He employed instead one part of garden soil and two parts of rabbit faeces, mixed with water and allowed to dry in the air, with repeated stirring. He further modified the original techniques by confining the gravid females in groups of 20-30 in earthenware pots 15 cm. in diameter and 5 cm. in depth.

Christophers maintained his cultures at 24-26° C. and Whittingham his at about 26° C., but the majority of workers appear to have kept them at bench-temperatures, which varied according to the locality and the season of the year. Ashner, in his experiments, maintained the developing larvae at a temperature of 30-32° C., which he stated to be the optimum for their development.

Since Ashner constantly maintained his cultures in an incubator, the larvae presumably completed their life-cycle in darkness; and Barretto (1942) has shown that adult females will live and oviposit in the dark. These observations suggest that the entire life-cycle of *Phlebotomus* can take place in the absence of daylight—a suggestion confirmed by our work and commented on later in this paper.

Sufficient has been said to indicate the type of apparatus used and the meteorological conditions maintained for breeding sandflies under artificial conditions, and it would be pointless to enumerate further the slight modifications used by subsequent workers. It is necessary, however, to consider previous observations regarding the food-requirements of the larvae and adults. As regards the larvae, it is probable that these may be raised to maturity on a wide variety of food-substances, but it is sufficient to state that the food principally used has been the faeces of rabbits or goats with or without the addition of garden soil and dried blood (Smith, 1925; Christophers *et al.*, 1926; Shortt *et al.*, 1926; Ashner, 1927; Theodor, 1934).

As regards the food-supply for the maintenance of adults in captivity it is, of course, well known that the females can be induced to lay eggs only after a blood-meal. In the vast majority of techniques used by previous workers the sandflies have been fed on the arms of human volunteers, and, although Adler and Theodor (1935) and Feng and Chung (1939) induced them to feed on dogs, they did not use this technique as a method of maintaining the strain. On the other hand, Wanson (1942) fed *P. schwetzi*, *P. africanus* and *P. squamipleuris* on lizards, and successfully used these reptiles to maintain his colonies of sandflies. Smith *et al.* (1940) claimed that the life of *P. argentipes*, given a single blood-meal on a kala-azar patient, is prolonged if the insects are subsequently maintained on a diet of raisins in place of repeated blood-meals. Using this technique they found that an average of 50 per cent. of flies survived beyond the 10-day period. In our experiments

with *P. papatasi* no food other than blood was supplied, and a similar percentage of sandflies survived the period.

We may conveniently summarize the observations of previous workers by saying that, given a proper food-supply for the larvae and adults, the principal factor to be observed in the breeding of sandflies is the maintenance of a proper degree of humidity during the entire life-cycle of the fly. In the words of Shortt *et al.* (1926), 'Dryness is the one condition to be carefully avoided from the time the eggs are laid until the hatching of the adults.' And Adler and Theodor (1935) stated 'that whereas egg development was not influenced to any marked degree by relative humidity (eggs developed normally when the insects were kept at a relative humidity of 70%), the insects laid all or nearly all their eggs only when the relative humidity approached 100%.' In the case of the adults it is important that this high humidity be maintained without condensation of water-vapour, in which the flies may become caught.

PRESENT INVESTIGATION

The colony of *P. papatasi*, the maintenance of which we are about to describe, was started from larvae and pupae of laboratory-reared sandflies kindly sent from Palestine by Professor Adler and received in this country on October 18th, 1945.

Two techniques have been used by us, in both of which the entire life-cycle of the flies was passed in the dark in an incubator kept at 30° C., except for the few minutes each day when the cultures were examined in daylight. The first technique to be used was similar to that employed by Shortt *et al.* (1926), in which adult males and females, as they emerged, were placed in lamp-glasses, each of which contained a piece of white card as a resting-place. The lamp-glasses were closed at the narrower end by means of cotton-wool plugs and at the wider end by covers of organdie carrying small holes through which the flies were introduced. The wide ends of the glasses were covered during the intervals between the feeds by pieces of damp lint. Human volunteers served as a source of the blood-meal.

When ready to oviposit the females were transferred to the egg-laying chambers. Each of these consisted of a lamp-glass, the narrow end of which was placed inside a small earthenware pot surrounded by damp soil; the space between the glass and the sides of the pot was packed with damp cotton wool and the wide end of the container, as before, was covered with a piece of damp lint.

After oviposition had occurred the pots containing the eggs were covered over with damp lint and set on one side to await hatching. A small amount of food, consisting of one part of garden soil and two parts of rabbit faeces, prepared according to the technique described by Ashner (1927), was added to the pots shortly before the eggs were due to hatch, i.e., on the fourth or fifth day. The moisture inside the pots was regulated by adding small amounts of water to the earth in which they were embedded. It is difficult to define the exact degree of moisture necessary for larval development, but with a little practice it becomes possible to judge whether the pots are too wet or too dry simply by examining the larval food, which should be damp and crumbling but should show no appearance of shining wetness. As soon as the pupae appeared the pots were uncovered and placed in small cages from which the adults could readily be recovered.

As a result of the adoption of this technique we succeeded in establishing a colony of *P. papatasi* and in maintaining it through three generations. It soon became evident,

however, that some simpler technique would have to be devised, for, as the flies increased in numbers, so did the amount of time spent in feeding them and in transferring them from one type of container to another, until three or four hours of one person's time was being devoted daily to the maintenance of the strain. In formulating a new technique it was necessary, therefore, to devise a single piece of apparatus in which the flies could undergo the whole of their cycle of development, and to eliminate the human host as a source of blood-meals. In the technique eventually adopted, an attempt was made to copy the principle of the rodent burrow, which has been shown to be an important breeding-place of sandflies, while the drawbacks of feeding the flies on human volunteers were overcome by using baby rats as the source of the blood-meal. The technique finally devised and now successfully used is as follows.

The essential part of the apparatus is a block of porous cement (we used Keene's cement) measuring 6 in. in every dimension. This block is cast to enclose an elongate chamber, narrower at one end than at the other and having a removable roof. The

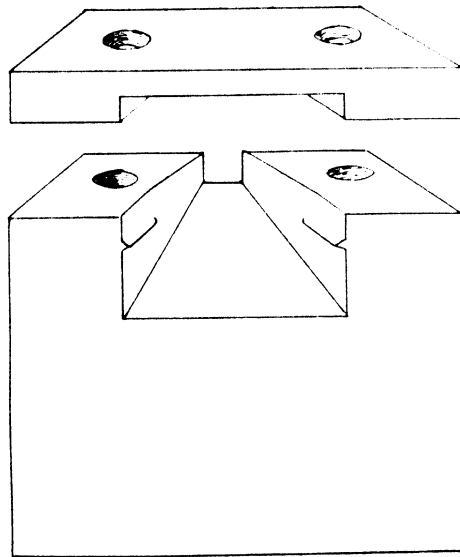


FIG. 1. Breeding-chamber.

chamber is open at both ends and is 6 in. long, $3\frac{1}{2}$ in. wide by $2\frac{1}{2}$ in. deep at the wide end, and 1 in. wide by 1 in. deep at the narrow end (fig. 1). Situated half way up the lateral walls of the chamber at the wider, or anterior, end are two projecting ridges, which, when the apparatus is in use, support a cork tray holding a baby rat.

Two cylindrical wells, 1 in. in diameter, and running vertically through the block to within 1 in. of the base, lie on each side of the chamber and serve as reservoirs for water. The proper degree of moisture can be maintained inside the chamber by filling up the water-reservoirs every two days.

When in use the block stands in the centre of a plywood base 19 in. in length and 8 in. in width, provided with grooved portions of wood on each side. The cages, which are used to close the chamber at each end, are carried on wooden slides, each consisting

of a vertical piece of plywood, which carries the cage, and a horizontal piece, which fits into the grooves of the plywood base. The larger of these two cages—that situated at the anterior end of the chamber—is provided with an organdie sleeve, while the posterior cage carries a small hole, through which the flies are introduced into the chamber. The slides carrying the cages press firmly against the porous block, and in order to render the chamber flyproof they are covered on the side against the block with felt (fig. 2).

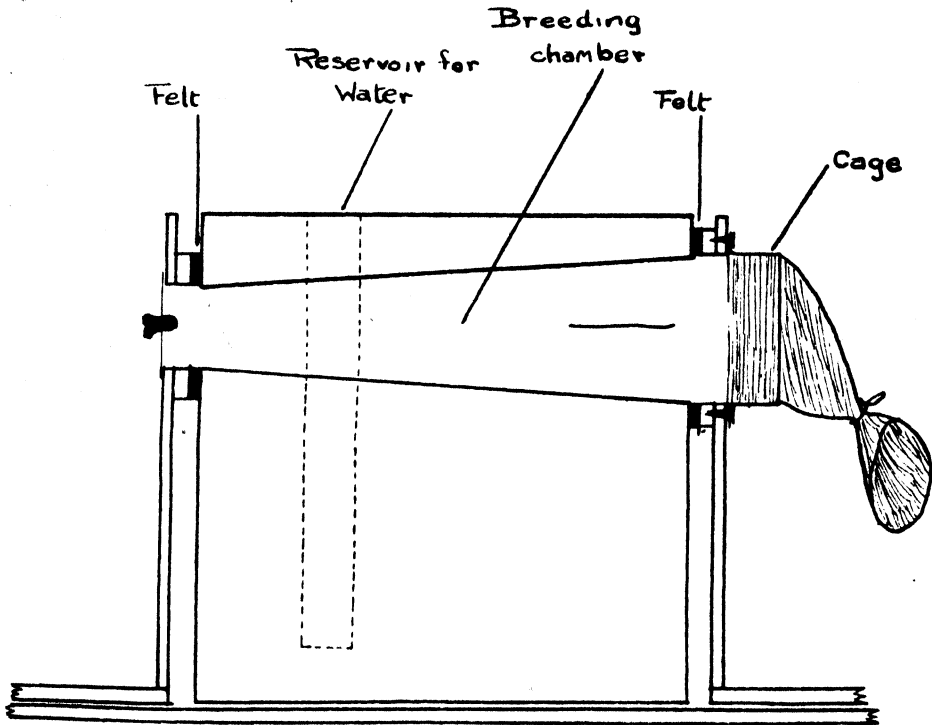


FIG. 2. Cross-section of breeding-chamber.

Prior to use, the entire block is soaked in water and afterwards placed on one side until the excess water has drained away. The floor of the breeding-chamber is then covered to a depth of about $\frac{1}{8}$ in. with food for the larvae, consisting of garden soil and rabbit faeces. The block is then placed in the incubator at 30° C. for 24 hours; at the end of this period the warm block is placed on the plywood base and the chamber is closed by sliding the cages into position and securing the slides by means of wedges (fig. 3). The adult flies are then introduced into the chamber through the hole in the posterior cage. Every morning the flies are given an opportunity to feed on a baby rat. The rat is placed on a small piece of cork, fastened to the end of a piece of wire about 5 in. in length, to facilitate introduction, and wide enough to rest on the ledge provided for this purpose. The animal is lightly restrained by covering it with mosquito-netting fastened to the cork with drawing-pins. The rat used should be less than three weeks old, since after this age the growth of hair prevents the sandflies from feeding readily.

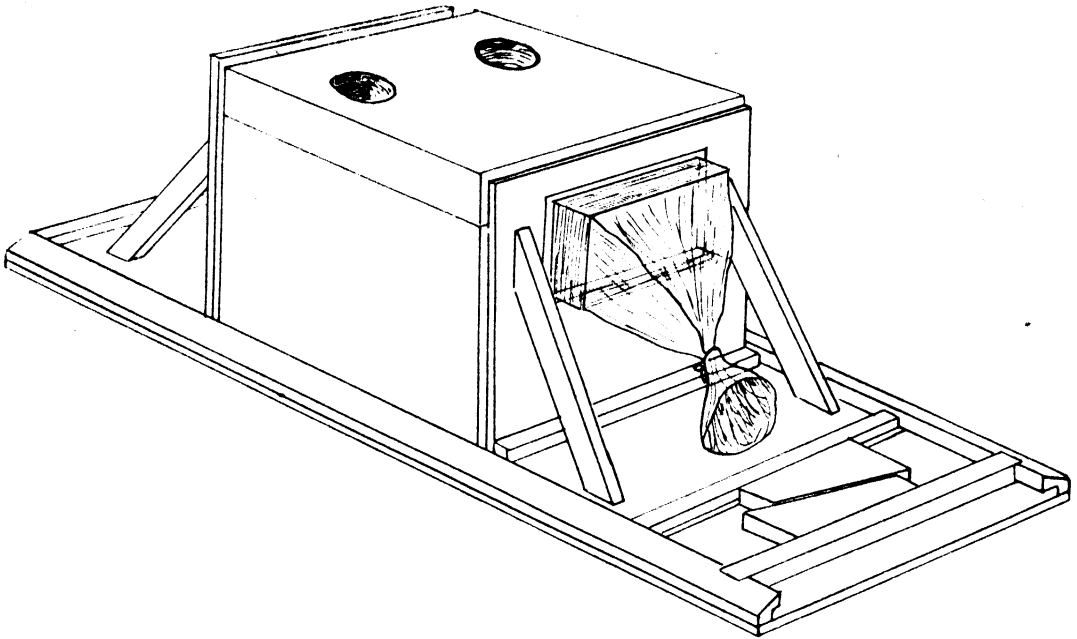


FIG. 3. Apparatus assembled for use.

RESULTS OBTAINED

By the techniques outlined above the original strain of *P. papatasii* sent from Palestine has been maintained in this country for a period of eight months, the flies, at the time of writing, being in their fifth generation.

The first technique which we have described gave the best opportunity for making observations on individual flies, and the details we are about to record were all made on flies kept under these conditions.

As regards the adult flies, no difficulty was experienced in inducing them to feed on the arm of a volunteer. As a general rule, apart from the first day after emergence and the two days preceding oviposition, the female fed during the first 20 minutes, or refused for that day. The females were given an opportunity to feed every day, but the number of blood-meals varied, as also did the days upon which they were taken; the majority, however, took four blood-meals, often on consecutive days, before their refusal to feed indicated that the time for oviposition was approaching. Of 48 female flies of which detailed records have been kept, one oviposited after one blood-meal, 13 after three blood-meals, 27 after four blood-meals and seven after five blood-meals. As regards oviposition, eight females oviposited seven days after emergence, 12 females after eight days, four females after nine days, 11 females after 10 days, five females after 11 days, and eight females after 12 days. Among 48 flies in which oviposition was observed, the maximum number of eggs laid was 66, the minimum three, and the average 19. The number most usually laid was between 20 and 30. The incubation-period in the case of 24 batches of eggs averaged six days. The majority of the larvae pupated after 25-30 days, but a proportion from the same batch of eggs always considerably lagged behind in development, a tendency, however, which did not appear to increase substantially during the winter, as was found by Theodor

(1934). The larvae generally pupated in the drier and more superficial layers of the food-material or on the sides of the containers. The average duration of the life-cycle from adult to adult was approximately six weeks, and the number of adults emerging, in relation to the number of eggs laid, varied from 28 per cent. to 57 per cent. of the possible yield.

It is difficult to assess the results obtained by the second technique described, as individual observations are much more difficult to make. At the time of writing, five breeding-chambers are in use, and in one of these the flies are now in the second generation. The flies feed very readily on young rats, and a proportion of them appear to oviposit readily in the food-material provided.

Results to date have been good, although they tend to be a little variable. From one chamber, in which 28 female flies were placed, 30 flies of the second generation have so far emerged. Examination of the food-material inside this chamber has revealed 103 larvae and 15 pupae. From another chamber, however, into which 42 female flies were introduced, only 36 larvae and 24 pupae were found. It should be noted, however, that the larvae hide among the food-material and are often quite difficult to find, so that these figures may not represent the total number present. The duration of the life-cycle is approximately the same as in the case of the flies kept according to the first technique.

DISCUSSION AND SUMMARY

The work of previous observers on the breeding of sandflies has resulted in the accumulation of a substantial literature relating to their natural breeding-places, habits and life-cycle and to their maintenance under artificial conditions. As a result of this combined knowledge, a method of maintaining colonies of the flies has been established, which has been so well tried and, in its broad principles, so generally adopted as to merit the description of 'classical.' Despite the success of this technique when employed in countries in which the flies naturally occur, it has not hitherto been found possible to establish and maintain a colony of *Phlebotomus* in this country, although the importance of the insect as a vector of disease undoubtedly merits the keeping of strains for research and teaching purposes.

By the adoption of the basic principles of this classical technique it has been possible to establish a colony of *P. papatasi* in Great Britain. The technique, however, is so time-consuming as to limit its use in this country if other than small colonies of the flies are to be maintained, and it was to overcome the disadvantages of the first or classical method that the second technique described in this paper was devised. Whereas the new method is perhaps not so productive as the first, this possible loss in efficiency is greatly outweighed by the simplicity of the technique, which gives a constant turn-over of sandfly material with a minimum of attention—a not unimportant consideration in institutions maintaining a large variety of insect strains.

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THE BIOLOGY AND MAINTENANCE OF *LIPONYSSUS BACOTI* HIRST, 1913, AND AN INVESTIGATION INTO ITS RÔLE AS A VECTOR OF *LITOMOSOIDES CARINII* TO COTTON RATS AND WHITE RATS, TOGETHER WITH SOME OBSERVATIONS ON THE INFECTION IN THE WHITE RATS

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During the past 25 years the control and treatment of the majority of insect-borne tropical diseases have advanced with a rapidity and success which renders all the more striking the lack of progress achieved in alleviating suffering due to certain tropical infections, which, though small in number, are nevertheless widespread and highly important. Pre-eminent amongst these are the various forms of filariasis, concerning which, although we know the cause and, in most instances, the vector, little has been accomplished in the way of control and almost nothing to justify confidence in the successful treatment of the disease. The latter failure is probably due to a number of causes, the most outstanding of which has been the lack of success in discovering a suitable laboratory animal capable of infection with a strain of filariasis well adapted for chemotherapeutic investigation.

This lack of a susceptible laboratory animal has proved, as it did for many years in the case of yellow fever, the chief stumbling-block in the path of success. At first sight it might not appear difficult to find a suitable host, since almost every genus of bird, mammal and reptile, not only in the tropics but also in the temperate zones, is parasitized by one or more species of filaria. Until very recently, however, no vertebrate host possessing all the necessary qualifications had been found, and the writers' own—though admittedly very limited—experiences during the past seven years may be cited as examples of the difficulties encountered.

Dogs infected with *Dirofilaria immitis* have been probably more extensively used in the chemotherapy of filariasis than any other animals, and at first it might be thought that they were admirably suited for this purpose, since the dog is, in certain respects, a suitable laboratory animal and the infection is easily transmitted to it by mosquitoes. A strain of this infection was maintained at the Tropical School at Hamburg, and by courtesy of Dr. Vögel we had arranged for infected animals to be sent to this country in September, 1939—an arrangement which was, of course, cancelled by the outbreak of war. Later, however, we came to the conclusion that this strain of dog filariasis was not likely to prove entirely satisfactory. In the first place the dog, although a good laboratory animal, is

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on the large side and relatively expensive, while the long incubation-period—some nine months—of the helminth infection renders it difficult to arrange for an adequate supply of infected material to be available at any particular date. We next considered birds, particularly the smaller cage-birds, which take up little room and of which many are comparatively inexpensive. A large number of species of birds, some of which occur in this country, are known to be infected with microfilariae. We have been allowed to examine the records of autopsies of birds made at the London Zoo, and we may quote the following figures as examples of the widespread nature of the infection: sugar-birds eight out of 14, cardinals eight out of 17, green singing finches three out of seven, and nutmeg finches two out of three. For laboratory purposes, however, it is necessary to work with a species which is either obtainable cheaply and in large numbers or will breed readily in captivity. Through the courtesy of the late Colonel S. P. James we obtained from Professor Brumpt's laboratory in Paris a Java-sparrow showing microfilariae in the peripheral blood. Of all the birds considered this seemed particularly suitable, since it is inexpensive, easily obtainable, small in size, breeds readily in this country, and is easily tamed. Various arthropods were fed on this bird, but no development to the infective form was obtained and eventually we lost the strain. More recently we have maintained various lizards and one species of frog (*Rana sphenoccephala*) infected with filariasis, and, although it is probable that one, at any rate, of these strains is transmissible by laboratory strains of mosquitoes, we came to the conclusion that these reptiles were unsuitable for chemotherapeutic investigations. Our experience, therefore, proved to be the same as that of other workers who failed to find a laboratory animal suitable for maintaining a strain of filariasis of a type which might be used for chemotherapeutic investigations. Our experience has taught us, however, that the essential requirements are as follows: a warm-blooded vertebrate host, easily maintained and easily bred under laboratory conditions, and susceptible to some form of filariasis sufficiently analogous to human infections to be used for screening anthelmintic drugs. Finally, of equal or even greater importance is the finding of a species of vector of the filarial infection of a type which can be bred and maintained in the laboratory.

The parasitization of the cotton rat (*Sigmodon hispidus*) with *Litomosoides carinii* has been known since the time of Chandler in 1931, and its more recent use for the preparation of typhus vaccine in America and in this country has shown that it is a comparatively suitable laboratory animal and breeds well in captivity. These facts were sufficient to warrant the belief that the cotton rat might prove the ideal animal for studying the chemotherapy of filariasis, and attention in America and in this country was at once centred on discovering the vector. A series of possible arthropod vectors was tried without success, until Williams and Brown (1945) published their discovery that *L. carinii* was apparently transmissible to the cotton rat by the tropical rat-mite *Liponyssus bacoti*. This discovery is, of course, a very important one, since it opens the way for the establishment of a strain of filariasis for the testing of various drugs likely to prove of value in the treatment of filarial infections in man and domestic animals. It is our opinion, however, that, before this can successfully be undertaken on a large scale, it is important that we should know not only the life-cycle of the parasite in the mite, but also how it is transmitted to the vertebrate host. In addition, it is essential to have a clear knowledge of how to breed and maintain the mite, and of its relationship to, and effects on, the vertebrate host. Without this knowledge, not only would it prove impossible to regulate the intensity of

the infection, but also, as the experience of other workers has shown, there is a grave danger of losing the strain of mites transmitting the infection, or of killing the vertebrate hosts by unnecessarily exposing them to too heavy infestations of the mites. It was with a view to eliminating some of these difficulties and permitting the maintenance of both vertebrate hosts and vectors on a large scale in the laboratory that the work described in the following paper was undertaken.

TECHNIQUES

With the detection of *L. bacoti* in the bedding sawdust of 10 infected cotton rats about a month after their arrival at the Liverpool School of Tropical Medicine, the work developed along two main lines. First, it was clear that the rôle of the mite as a vector would have to be studied in detail, and, secondly, it was desirable to attempt the transmission of the infection from cotton rats to white rats. Consequently, techniques developed in two directions, and it is convenient to discuss the techniques under two headings, namely, routine techniques and experimental techniques.

Routine techniques are those employed for maintaining strains of *L. bacoti* having unrestricted access to cotton and white rat hosts over a period of weeks, some of the hosts being the source of infection to the mites and the others being uninfected hosts exposed to infection.

Experimental techniques are those developed during the study of the biology of the mite and its rôle as a vector. Several of these have obvious possibilities as methods with which to modify or replace the present routine techniques.

ROUTINE TECHNIQUES

Earlier Methods. At the time of the detection of the mite the infected cotton rats were each accommodated in a wire cage 11½ in. by 3½ in. by 3 in., and five of these cages were placed in a fibre or zinc tray 4 in. deep containing a 2 in. deep layer of sawdust. Cotton fibre and wood-shavings were present in each cage as a nest for the rodent. The sawdust-trays stood over a water surround.

For some time this type and size of rodent community was maintained, but in one transmission-experiment a larger wooden tray was set up; the bedding-materials were as before, except that hay was substituted for the wood-shavings.

Disadvantages of Earlier Methods. In the absence of precise information on the percentage of infective mites likely to occur in a strain, it was considered that only by subjecting the rats to a heavy infestation of mites over a period of some weeks could transmission of infection to uninfected cotton or white rats be expected. This limited the frequency and the thoroughness with which soiled bedding-materials could be changed, and only periodic removals were made of portions of the bedding. In the course of time parts of the bedding became soiled with the excreta of the rats, and heavy cultures of tyroglyphid mites developed, together with maggots of *M. domestica* and *F. canicularis*. Cheyletid mites, some of which contained blood presumably obtained by predation on the blood-sucking mites, occurred in limited numbers, and occasionally a few free-living and other blood-sucking gamasid mites were observed. Such an environment, particularly the excessive dampness, was unsuited to *L. bacoti*, and the presence of the undesired fauna in soiled materials, together with a migration of *L. bacoti* into the water surround, indicated that the method required alteration, in the interests both of the vector and of the rodent hosts.

Another unsatisfactory aspect of the trays containing both cotton and white rats particularly concerned the question of the transmission of the infection to the white rats. It was apparent that, in the large community of mixed cotton and white rats described above, the relative suitability of the two types of rat as hosts resulted in a concentration of *L. bacoti* on and around the cotton rats. Shelmire and Dove (1931) found it necessary to fit collars to albino rats to prevent them from ridding themselves of *L. bacoti* mites. A more convenient method was clearly required to ensure that sufficient infective mites would occur on the white rats to produce infection.

Briefly, it may be stated that communities of several cotton and white rats exposed to unrestricted infestation for several weeks are not satisfactory, as the density of the vector is constantly subject to the adverse effects of at least three factors, namely, periodic clearing out of bedding, excessive moisture in soiled bedding, and the predation of the white rats. It was evident to us that an entire strain of *L. bacoti* could be lost by relying on the methods described above.

Although at present no fundamental change has been made in the environment provided for the rats and mites, we have instituted what we call the single unit, as a means of minimizing the disadvantages of the larger communities.

The Single Unit. A single unit consists of a standard biscuit-tin, 9½ in. by 8 in. by 8 in., with the usual turned-in flange of about ¼ in. at the top, the tin being mounted on legs over a water surround. A layer of sterilized sand and earth, or simply sand, about 3 in. deep, is laid in the biscuit-tin, and on top of this is placed a 6 in. cubic wire cage. Cotton fibre has been discarded, as it adds to the work of isolating mites from the unit, and hay also has been abandoned, since it rots and encourages tyroglyphid mites. Wood-shavings are now packed in the space between the cage and the side of the biscuit-tin. Some, but not all, of the rats pull the shavings into the cage. Normally, one cotton rat or one white rat is accommodated in the cage of a single unit.

The single-unit method has also been employed to maintain a strain of uninfected mites on an uninfected cotton rat, but, in view of the experimental techniques discussed below, such a strain—at least in filariasis work—is not essential.

Advantages of the Single Unit. Although the single unit in its present form will develop the same disadvantages as the larger trays, there are a number of points which represent practical advances over the earlier methods: fly-proof lids are readily fitted to a biscuit-tin; dampness, with its concomitant outbreak of tyroglyphid mites, does not develop so readily in the sand base; and the mites do not tend to climb up to or over the flange at the top of the tin.

The mite-population, if considered excessive, can be depleted by leaving the mites without a host for 14 days. On the other hand, the population may be usefully distributed over a series of uninfected rats by placing them separately for 3–4 days in the unit and then accommodating them in a similar clean unit, or, if it is so desired, by placing them over water for at least three days in order to shed their mites before being set aside as 'clean' potentially infected animals. Alternatively, the material itself may be subdivided amongst several new units.

A particularly important point is that by using single units transmission can be more certainly achieved, since the mites necessarily swarm on to any new host.

Finally, the single unit is convenient to handle for the periodic removal of soiled bedding from or immediately under the cage. It is proposed to introduce into each

biscuit-tin a zinc tray 6 in. by 6 in. by $1\frac{1}{2}$ in. deep, and to sink this into the sand below the cage. The contents of this tray should prove unsuitable for *L. bacoti*, and would be discarded when necessary without materially affecting the mite-population. The substitution of oil for water in the surround would be an improvement.

Disadvantages of the Single Unit. There is one particular disadvantage common to the earlier communities and the single unit, and that is that, although a short exposure of a rat to a large mite-population lessens the possibility of the death of the rat due to excessive infestation, the method may well lead to an intensity of filaria infection which may later prove to be lethal to the host. There is some indication that this can occur in cotton rats exposed over many weeks to considerable infestation and constant re-infection. Thus, one cotton rat which died after such an exposure contained an uncountable mass of tangled immature and adult worms in the pleural cavity, and the number of microfilariae in the peripheral blood, counted against white blood-corpuscles, was estimated at 3,700 per c.mm. This is a heavy infection when compared with counts of 200–1,000 per c.mm. in cotton rats examined by Brown and Williams (1945).

Rats with this intensity of infection are now kept free of mites and used as sources of infection for experimental work.

It remains to be pointed out that, since by the method of mass-infestation other arthropods may occur, including, as we have noted above, occasional specimens of blood-sucking mites other than *L. bacoti*, it follows that the transmission of the worm may, in part, be due to arthropods other than *L. bacoti*. However, the development of the filaria parasite in *L. bacoti* as described by us (p. 247), and the infection obtained by us in the case of two white rats, not in contact with infected animals, but infested with 200 *L. bacoti* from infected cotton rats (p. 250), together with the reports by Williams and Brown (1945, 1946), indicate that such a possibility is not of major significance.

The techniques described in the next section provide methods of ensuring the identity of the vector and of obtaining the information required if the intensity of the infection in the vertebrate is to be controlled.

EXPERIMENTAL TECHNIQUES

The strains of uninfected *L. bacoti* used in our experimental work were bred from the eggs of gorged females, the females being obtained by placing an infested rat over water or by isolating them from samples of bedding-materials, using the apparatus described by Bertram (1946). It will be clear from what follows that either infected or uninfected females may be used as the source of the eggs.

Storage of Adults for Oviposition. The adults are kept in 2–3 in. lengths of $\frac{1}{4}$ in. wide glass tubing plugged at both ends with tightly packed cotton wool. A strip of filter-paper covering about a third of the circumference of the tube is placed between the plugs. It has been found convenient, when transferring the mites, to stick one end of the tube in a mass of white plasticine, from which it projects at an angle of 45° , the plasticine being surrounded by water or by a wide area of white paper. A white plasticine plug is kept over the opened upper end of the tube throughout the manipulations, except at the moment of adding a mite. Individual mites are lifted on the point of a damp paint-brush from the water-tray or from the collecting-chamber of the apparatus referred to, and are first placed momentarily on filter-paper to drain off excess moisture. The mite is then quickly deposited about $1/10$ in. within the glass tube, and the tube is sharply tapped until the mite falls

to the lower end of the tube. The plasticine plug is replaced, and a second mite is picked up on the brush and dealt with in the same way. Up to about 50 gorged females is a convenient number to accommodate in one tube. After the last mite has been put in, the filter-paper lining is pulled out until the moist part where the mites were deposited protrudes. This portion is cut off and the remainder pushed down into the tube, which is then sealed with another wool plug. Dark cotton wool is a convenient background against which to detect the eggs. It is essential to avoid leaving moisture in the tube.

Segregation of a Pure Strain of Protonymphs. Tubes of ovigerous females are stored in an incubator at 25° C.

In 48 hours the majority of the eggs will have been laid, and between 200 and 400 may be expected from 50 females collected at random. Before the eggs hatch one plug is removed and the open end of the tube is quickly joined, by a strip of adhesive tape, to a similar length of tube, across the end of which there is a membrane of no. 8 bolting-silk, the farther end of this tube being firmly sealed with cotton wool. The joined tubes are returned to the 25° C. incubator, and in a further period of about two days only active protonymphs and adults are present. A number of the protonymphs will have wandered through the membrane into the new tube, and the remainder are now driven through it by passing a heated sheet-tin ring slowly along from the wool plug of the tube containing the adults until the ring overlaps the adhesive tape. The tubes are now separated, the membrane is torn off, and the tube of protonymphs is sealed with a firm plug of cotton wool. This method kills the adults and any late larvae which may be present, but it has proved a rapid means of obtaining a pure strain of protonymphs. The nymphs should be fed about the fourth or fifth day after moulting from the larva.

Feeding of Protonymphs. Full engorgement of the protonymphs has so far only been obtained by giving the nymphs free access to a cotton rat for a period ranging from at least 12 up to 24 hours. The rat is placed in a wire cage, 4 in. square by 2 in. deep, set in a tin tray 5 in. square and 2 in. deep, the sides of the tray being turned in for about $\frac{1}{4}$ in. at the top to form a horizontal flange to minimize the escape of mites. A layer of about 20 gm. of sawdust is placed in the tray. The tray rests on a wire tripod about 8 in. high, and, to prevent the rat from upsetting the apparatus, wire strands pass from the cage to the tripod legs. The tripod stands in a water surround.

The protonymphs are released by removing one of the cotton-wool plugs of the storage-tube and by tapping the tube vigorously until the mites fall on the rat. At the end of the allotted period the rat in its cage is removed from the sawdust-tray and isolated on another wire tripod over water.

The sawdust in the tray is examined for gorged mites by spreading it over filter-paper or by treating it in the apparatus used for sampling bedding-materials.

The water surround under the rat requires examination for fallen mites for a period of 2-3 days, and, even after 12-18 hours of exposure of the rat to the mites on the sawdust-tray, the majority of the gorged nymphs are recovered from the rat during its period of 2-3 days over water. Approximately 50 per cent. of the nymphs released have been recovered in preliminary trials of the method.

The gorged nymphs are transferred to tubes of the type used for the storage of ovigerous females. Up to 50 nymphs is a convenient number for one tube.

The Production and Maintenance of Adults. Tubes of gorged nymphs are stored at

25° C., and within 2-3 days two moults are completed, without the need for a further blood-meal, resulting in adult males and females.

Two methods have been employed for feeding the adults :

1. The first method is that already described in connection with the protonymphs, except that the rat is exposed to the mites for a shorter period on the sawdust-tray before being transferred over water. At least three hours should be allowed. Collections are made over a period of 2-3 days, and about 80 per cent. of the females released are recovered. The adults are collected and stored as before, until they appear to require a fresh meal. In general, a second meal is not necessary for 4-6 days, by which time the eggs have been laid and the females have become flatter. In transferring the adults on to a cotton rat for a second meal it is best to tap the adults out on to water and from there to lift them on a brush on to the rat. Otherwise some protonymphs derived from the eggs may establish themselves on the rat and may lead to confusion at a later stage. The process is repeated at intervals as necessary. Although Dove and Shelmire (1931) devote little space to techniques, it is clear that they released mites to feed on guinea-pigs placed over water.

2. We have used the second method so far only with white rats, about three months old, as hosts. The rat is confined in a perforated zinc cylinder, through the end-piece of which the tail projects. An adhesive-tape strip is bound round the base of the tail and fixed to the cylinder, to prevent the tail from being withdrawn by the rat. A mass of white plasticine is now moulded firmly round the base of the tail, so as to form a ring about 1 in. in diameter. The adult female mites are now transferred to a feeding-tube made from the lower 3 in. piece of a $\frac{1}{2}$ in. wide test-tube, the open end being abruptly tapered to a diameter equal to that of the rat's tail at the level of the plasticine ring. The mites are most easily transferred to the feeding-tube by first removing one cotton-wool plug from the storage-tube, which is then inverted over the feeding-tube and held tightly against the narrow opening. It is convenient to fix the feeding-tube vertically on a plasticine base surrounded by water. The storage-tube is tapped vigorously, and the few mites which fail to fall into the feeding-tube are transferred individually on a damp brush. The feeding-tube is then temporarily sealed with plasticine. Before applying the feeding-tube to the rat's tail, the tail is rubbed along a length of 1-1½ in. on one surface with a razor-blade held transversely and almost vertically to the long axis of the tail. This should be done only on the surface which is uppermost when the tail is at rest, and only to the extent of removing the scales with, at the most, a few minute points where either blood or serous fluid begin to exude. The plug is then removed from the feeding-tube and the scarified tail is slipped into it until the open end of the feeding-tube is pressed firmly into the plasticine ring on the tail. The tail should rest on the lower surface of the tube with the scar-line uppermost. A bench-light is placed about 8 in. above the feeding-tube to provide warmth which at least keeps the mites active so that the chances of their all finding the scarified zone are greater. Mites, if ready, may feed on the scarified area within a few minutes, and feeding occurs readily both in direct light from the lamp and when the mites are shielded from it. After engorgement, the feeding-tube is removed and the mites are tapped out on to a large area of filter-paper or water, and are transferred once more by means of a paint-brush to the usual type of storage-tube.

It is pointed out that, if the scarification is excessive and produces readily visible exudations of blood and serous fluid, the mites tend to take up more serum than whole

blood and to stick in the serum, particularly if this has flowed on to the glass. If after about one hour a number of mites in a batch have not fed, the tail is removed from the tube, cleaned, and reinserted for a further period. About 25-30 females to a feeding-tube is a convenient number to manipulate. This technique has proved unsuccessful for protonymph feeding, and so far no attempt has been made to keep large numbers of males alive.

THE LIFE-CYCLE AND HABITS OF *LIPONYSSUS BACOTI*

(PLATE VIII, FIGS. 1-12)

GENERAL

The strain of *L. bacoti* which provided the material for study was derived from cotton rats infected with the filariid worm *L. carinii* and sent from Canada to the Liverpool School of Tropical Medicine. At the time of their arrival no mites were apparent, but about a month later *L. bacoti* was detected in the bedding-materials of the rats. Having read the report by Williams and Brown (1945) on the presence of developmental stages of the filaria worm in *L. bacoti*, we collected mites at random from the bedding-material and confirmed the findings of the American workers. It soon became apparent, however, that a knowledge of the life-cycle of the mite was necessary as a basis for developing techniques for maintaining and manipulating the mites in experimental work, and the account which follows describes the results of the work undertaken.

The medical literature contains many references to *L. bacoti* as a cause of dermatitis and a few references to it as a possible vector of endemic typhus, but data on the life-cycle are limited. Hirst (1913) first described the female, and later (1914) illustrated and described the female, male and unfed protonymph. The egg and larva are described by Holdaway (1926), who gives an account of the habits of the larva, the unfed protonymph and the female. Olson and Dahms (1946) give an account of the adverse effects of mass-infestations of *L. bacoti* on guinea-pigs, hamsters, white rats and mice, together with some observations on larval and adult habits.

The fullest report of the complete life-cycle is available in a series of papers by Shelmire and Dove (1931) and Dove and Shelmire (1931, 1932), who investigated the subject while studying the potentialities of *L. bacoti* as a vector of endemic typhus in America. These authors do not, however, refer to the non-blood-sucking hexapod larva first noted by Holdaway (1926), or to the non-blood-sucking deutonymph which is recorded in the present paper. The present account of the life-cycle of *L. bacoti* differs in several respects from that given in the joint papers by Shelmire and Dove but conforms closely to the life-cycle of *L. nagayoi* Yamada as reported upon by Yamada (1931) and Ohmori (1935, 1936, 1937).

Briefly, we have found that the life-cycle of *L. bacoti* consists of the following stages :

1. The blood-sucking adult female and male.
2. The egg.
3. The hexapod non-feeding larva.
4. The octopod blood-sucking protonymph.
5. The octopod non-feeding deutonymph.

Each immature stage moults once, and since the female lays viable eggs after the first adult meal the cycle can be completed from egg to egg with only three ecdyses and

two blood-meals. With the mites stored in dry conditions in the dark at 25° C., except when feeding on cotton rats in a moderately dry and well-lighted laboratory at 20–22° C., the life-cycle required 10–12 days for its completion.

The descriptions given below of the various stages are concerned not with detailed morphological features, but mainly with aspects of the appearance or activity of the mite such as are likely to be of value to those concerned with the handling of this species in experimental studies, while the photographs (Plate VIII) are intended to give a comparative impression of the appearance and sizes of the different stages. The references to descriptions of systematic value which are given in the appropriate sections of this report are not exhaustive, but have been selected either as providing detailed descriptions or for the illustrations which they contain.

THE ADULT FEMALE (Plate VIII, figs. 10, 11)

Descriptions of systematic value are given by Hirst (1913, 1914), Ewing (1923) and Finnegan (1945). The size of females as recorded by them varied between 0.78 and 0.95 mm. long by 0.56 mm. broad, according to the degree of distension.

Digestion in the Gorged Female. We have found gorged specimens up to 1.5 mm. in body-length, the entire ovoid body, except for the legs and the part just behind the capitulum, being filled by the distended gut diverticulae, which may be bright red to black in colour according to the amount of residue present from previous blood-meals. Within a few hours of a meal a small white Y-shaped mark develops on the dorsum, as well as a white area ventrally at the anus, due to accumulations of the crystalline excrement of the Malpighian tubes. As the meal is digested over the next 24 hours or so, a pyriform translucent area, sometimes bilobed behind, appears in the forwardly directed fork of the Y-mark. This is associated with the development of the eggs, which in general begin to be laid on the second day after the blood-meal. Over a period of 2–3 days a female will lay all her eggs, becoming progressively less distended and markedly broader at the hind end than in front. When oviposition is completed the Y-mark and anal area persist, but the translucent area, which is apparently due to the eggs showing through the cuticle, disappears. Digestion of the blood-meal continues, and about the fourth to sixth day the female is usually markedly flattened and more oval, the diverticulae are brownish, the Y-mark and anal spot have become densely white, and the gut pattern, as seen in a starved specimen (Plate VIII, fig. 10), becomes apparent. At this stage the female is about 0.7 to 0.8 mm. in length.

Excretion (Plate VIII, fig. 12). Throughout this period of digestion the mite discharges excreta at random from the anus, consisting of white deposits often containing brown or black pigmentation from the gut.

Feeding. The females take up approximately 0.4 c.mm. of blood in one engorgement. They feed through delicate skin, but appear to be rather superficial feeders in that they are easily disturbed when feeding, particularly before distension is obvious, and they then remove their mouth-parts from the host, move away rapidly, and may not resume feeding for some time. They are readily attracted to scarified skin through which the host's blood is apparent or where a break in the skin has produced an oozing of blood or serum—an observation which has been utilized in inducing the mites to feed on the scarified tail of white rats. In certain of the transmission-experiments involving white rats, adults have been observed engorging on the tail at scars caused by notoëdric mange.

The readiness with which females will engorge is naturally subject to various factors, and the results observed in a number of experiments demonstrate the varied habits which the mites show in this respect.

Observations on the Feeding of Females on the Scarified Tail of a White Rat

The apparatus described on p. 234 was used, and 26 females, starved at 25° C. since moulting from the deutonymph 48 hours earlier, were released in the tube. Within three minutes 17 females were feeding on the scarred area, and six of these were fully engorged and bright red and had left the tail within 10 minutes. By the end of 30 minutes 18 females had engorged, and in two hours the whole batch was replete and wandering about in the tube. This first meal appeared to be digesting very rapidly, and a second meal was made available in the same way 30 hours later. Within 20 minutes all but one female had fed to repletion. When a third meal was offered four days later, 16 of the females began feeding within 15 minutes and all but one were engorged in one and a half hours. On the occasion of the fourth meal, after a further period of four days, only two mites fed in the first hour, four more in the next hour, and seven in the last half hour of a two-and-a-quarter hour period in the tube with the rat's tail. Of the remainder, seven mites were unfed, three were partially engorged, and one was lost.

The experiment shows that where a suitably delicate skin is available females may engorge within 10 minutes, and that, with increased age, mites of the same batch develop variations in their tendency to feed. This trend is presumably related to variations in the physiological state of the mites after a number of blood-meals.

Observations on the Feeding of Females Released on a Cotton Rat

The second type of experiment approximates to more natural conditions, as the mites were released on a cotton rat in the manner described on p. 233. In the particular experiment recorded in Table I the adults, which included males and females, had been starved at 25° C. since moulting three days previously from the deutonymph.

Three hosts were provided. The first host was placed with the mites on the sawdust-tray for one hour, and thereafter the rat in its cage was isolated over water. The sawdust was examined for mites, and periodic collections were also made of the mites dropping from the rat into the water. A second host was placed on clean sawdust, and males and such unfed or partially fed females as fell from the first host were transferred at each collection-period to the second host. Both hosts were kept in this way over night, one over water and one on sawdust. The second host was then placed over water, the sawdust examined, and a third host set up on clean sawdust.

The results are given in Table I. The table includes data for the males, and a discussion of this part of the table is found in the appropriate section below.

It can be seen from Table I that, of approximately 160 adults released in the experiment, 86 females and 64 males (150 adults) were recovered from the first host in varying stages of engorgement or unfed within 25 hours. After 24 hours of freedom to feed on one or two hosts 57 females had engorged (66 per cent.), 42 remaining on the first host for 1-21 hours and 15 requiring two hosts. A further 17 females required two hosts, and one female three hosts, to engorge within 48 hours. Of the 86 females known to have been present in the experiment, 75 attained repletion in 48 hours (approximately 88

per cent.). Females unaccounted for in recoveries from the second host amounted to 10 per cent.; two females were not released for feeding on the third host (3 per cent.).

TABLE I

Showing the number of hosts and the time required for *L. bacoti* adults to attain complete engorgement when free to feed at will on cotton rats. About 160 adults were used, and the period during which each cotton rat was available was as follows

Host	Period on sawdust after commencement of experiment	Period over water after commencement of experiment	Total period as host in experiment
1st	1st hour	2nd-25th hour	1st-25th hour
2nd	3rd-23rd hour	24th-50th hour	3rd-50th hour
3rd	26th-48th hour	49th-95th hour	26th-95th hour

Mites recovered from hosts

Host	Hours after commencement of experiment	Females			Males	Total adults
		Gorged	Partially fed or unfed	Total		
1st	1st*	1	0	1	1	2
"	2nd	4	2	6	3	9
"	3rd	8	11	19	8	27
"	5th	17	19	36	41	77
"	8th	11	9	20	10	30
"	21st	1	3	4	1	5
Total mites from 1st host in 25 hours ...	—	42	44	86	64	150
Total mites transferred to 2nd host ...	—	—	44	44	64	108
2nd	23rd*	15	0	15	0	15
"	25th	10	0	10	22	32
"	27th	3	3	6	4	10
"	48th	4	0	4	14	18
Total mites from 2nd host in 50 hours ...	—	32	3	35	40	75
Total mites transferred to 3rd host ...	—	—	1	1	26	27
3rd	48th*	1	0	1	10	11
"	77th	0	0	0	3	3
Total mites from 3rd host in 95 hours ...	—	1	0	1	13	14

* Indicates collection from the sawdust under the host.

The percentage loss of females in the whole experiment would be between 10 and 20 per cent.

It would appear, therefore, that under natural conditions females may take several hours to become replete, and that, over a period up to 48 hours, they may remain in the fur of one animal or drop partially fed or unfed from the host and complete the meal on a second or third animal. There is no reason to doubt that the process may be repeated, involving more hosts before engorgement is attained.

A comparison of the two types of experiment suggests that the delay in procuring a full meal on one or more hosts, which occurs under the more natural conditions, may be due to the time required for mites to locate scars or suitably delicate skin, together with disturbances whilst feeding as a result of the activity of the host.

The results of the experiment using the scarified tail demonstrated that the female mites will feed readily 48 hours after becoming mature, and that, apart from the second meal which may be necessary within 48 hours of the first, an interval of at least four days is required for the utilization of subsequent meals. Table II shows that six days may be allowed for digestion.

Dove and Shelmire (1931) collected gorged females after 1-3 hours' exposure on guinea-pigs over water, and noted that the females on engorging may remain on the host for several hours before dropping off. They use the term 'piecemeal feeding' to describe the habit of the mite in obtaining a meal of blood from several places on man, and suggest that this habit is due to the disturbance of the mite by the human host or to the fact that man is an abnormal host. Our observations show that 'piecemeal feeding' occurs on cotton rats, which may be presumed to be normal hosts.

Survival. Females kept at 25° C. after moulting from the deutonymph will survive unfed for 10 days. With blood-meals provided whenever it seemed necessary (about the fourth to sixth day) females have been kept alive for 33 days.

The longest length of life of a female recorded in the present study is 49 days, during which the mite had three opportunities to feed on a rat's tail, the last occasion being 39 days before death. The mite had been kept at temperatures ranging from 18° to 25° C.

The relationship of the blood-meals to oviposition is discussed later in the text.

Humidity. Critical observations have not been made on the effect of humidity on the females, but it is clear from general observations on routine stocks and on mites kept in storage-tubes that a damp surface or material is unsuitable. It has been found that minute condensation droplets of moisture in a storage-tube leads to inactivation and death, and that a similar result appears to follow when the mite is held in a film of water.

It is interesting to note, however, that mites falling from rats into a clean-water surround survive two days of complete immersion and five days if floating on the surface. They show little ability to travel on or under water.

Motility. The heat of a hand or of a bench-lamp readily activates the mites, which are usually rather inactive when first removed from the dark 25° C. incubator. Light appears to keep the mites in a state of unrest.

Females, when ready for a meal, are very active and can run at a speed of about 8 in. per minute, waving their fore-legs like antennae. They will burrow into cotton wool or between closely apposed surfaces. Although fully gorged females can move rapidly and even climb a vertical glass surface like unfed stages, they are generally less active than

hungry mites. During oviposition they stand over the batches of eggs, occasionally tapping the eggs with the palps or waving fore-legs.

THE ADULT MALE (Plate VIII, fig. 9)

The male was first illustrated and described by Hirst (1914), whose descriptions have since been repeated or amplified by various authors, including Ewing (1923), Willmann (1939), and Finnegan (1945).

The length of the body of the male is about 0.4–0.5 mm., and the dorsum is covered by a large chitinized plate. In outline they are elongate, oval, and more pointed at the hind end than the female. The colour of the body and the gut pattern in the male resemble those of the female. They also run rapidly, waving their fore-legs in front in the same manner. Males do not take a large meal of blood, despite long periods on the host, and at the most show only a slight degree of distension, together with a barely detectable red coloration of the gut diverticulae. From Table I it is seen that 63 males were recovered either unfed or slightly fed within eight hours of being placed on a cotton rat, and that 40 of these were later recovered from a second host and still showed no increased distension. Of 26 given a further opportunity to feed on a third animal, 13 were recovered without marked signs of engorgement. Thus, even after opportunities to feed on three hosts over 48 hours, no males were distended markedly with blood. Records of survival periods for the male are limited, but a male which had emerged from the deutonymph survived unfed for 11 days at 25° C.

MATING

It seems probable that a male may copulate several times with different females, as Yamada (1931) records for *L. nagayoi*, and, in view of the hours spent by both sexes on a host, copulation probably occurs at random on and off the host.

A male and female, which had emerged respectively 24 and 48 hours from the deutonymph stage, were placed together in a small glass tube plugged with cotton wool. Both mites ran freely in the tube, but within one minute they had come in contact and began to copulate, the process being completed within two minutes. During copulation the female came to rest, and the male climbed from behind on the underside of the female and brought his capitulum to the level of the female genital opening. The male capitulum was flexed towards and, apparently, into the vulva, but details of what occurred could not be seen. The process resembled that noted for *Ornithodoros* ticks by Nuttall and Merriman (1911), although no spermatophore is left attached by the male to the female orifice in *L. bacoti*.

OVIPOSITION

The eggs, which are broadly ovoid and translucent, and measure 0.35–0.36 mm. long by 0.22 mm. wide, were first described by Holdaway (1926), and we have observed that there is a variation in size similar to that found by Ohmori (1936) in *L. nagayoi*. The same author reports that unfertilized eggs produce males; in *L. bacoti* we have confirmed that parthenogenesis occurs, but we have not examined the question in relation to sex production. In our examinations the number of non-viable eggs is small, only three out of about 400 laid by females collected at random from stock strains failing to hatch.

Frequently eggs are laid singly, but females tend to lay them in groups, often where closely apposed surfaces in subdued light occur. In storage-tubes batches have been laid on cotton wool and plasticine plugs, on black cloth and filter-paper, in sawdust, or on the glass surface of the tube itself. As many as 200-300 eggs may be found, forming a uniform patch at one point on the glass. Examination of the fur of two dead infested cotton rats from routine stocks failed to show the presence of eggs on the rats, and it is apparent that oviposition takes place in the bedding-materials, although we have failed to detect them in a number of random samples.

It has already been mentioned that the females stand over the eggs, but there is no evidence to suggest that this is essential to the hatching of the eggs.

A female will lay a series of batches of eggs throughout her life, each batch being preceded by a blood-meal. A few observations have been made on the number of eggs per female per meal for females kept separately in tubes at 25° C. and fed periodically on a scarified rat tail. The results for an unfertilized and a fertilized female are shown in Table II.

TABLE II

Showing the food-requirements of and oviposition by one unfertilized and one fertilized female *L. bacoti*, maintained on the scarified tail of a white rat, for periods of 24 and 28 days respectively

♀	Length of ♀ life	Age of ♀ at mating	Age of ♀ at blood-meals	No. of eggs per blood-meal	Interval between meal and oviposition	Duration of oviposition period	Cause of death
1	24 days	No fertilization	4 days 10 " 13 " 19 "	6 12 2 Nil <hr/> Total : 20	2-5 days	2-3 days	Apparently starvation
2	28 days	2 days	2 days 8 " 13 " 18 " 21 "	1 6 1 5 7 <hr/> Total : 20	2-4 days	2-3 days	Apparently starvation

It is considered unlikely that 20 eggs is a maximum number. The result shown in the table suggests that fertilization did not influence the course or duration of oviposition.

In a further series of seven fertilized females which were fed once only and died 4-6 days later, an average of four eggs (minimum three, maximum six) per female was laid 2-3 days after the meal.

In dissections of females collected at random one full-sized egg and up to four additional developing eggs may be clearly identified. Occasionally, an egg showing complete larval formation is found in dissections.

The eggs adhere lightly to whatever surface they are laid on, and the larva emerges through a longitudinal slit in the upper side of the egg (Plate VIII, fig. 1).

THE LARVA (Plate VIII, fig. 2)

A description and figure, together with notes on the biology of the larva, were first given by Holdaway (1926), and Olson and Dahms (1946) refer also to its habits. In the papers by Shelmire and Dove the word 'larva' is used in a general sense, and is clearly applied by them to the active blood-sucking protonymph. They do not differentiate the hexapod larva from the octopod nymph.

The hexapod larva is little larger than the egg, and is ovoid and translucent white in colour. At 25° C. the eggs hatch in about 30 hours, and the larvae, which can walk slowly, although in an ungainly way, leave the shell but remain close to it. They do not feed, and in about 24 hours they cast their skins and become protonymphs. The larval moult has the appearance of a crumpled roll of skin, from which two of the legs protrude in a horn-like manner at one end (Plate VIII, fig. 3).

THE PROTONYPH (Plate VIII, figs. 4-7)

The protonymph was first figured and described by Hirst (1914), and, amongst others, again by Finnegan (1945). Holdaway (1926) gives a good description of its appearance in the living state and of its biology when unfed.

In random batches of protonymphs minor differences in size are apparent. Hirst (1914) gives the length of the body as 0.4 mm., whilst Holdaway (1926) records sizes from 0.32 mm. long by 0.20 mm. wide up to 0.5 mm. long by 0.27 mm. wide.

The nymph is translucent white, and is rather slow in its movements for several hours after the moult, but it acquires an ochreous tinge later and becomes active, running very rapidly, after the manner of the adults, and tending to burrow into cotton wool or between apposed surfaces. At this stage it is flat and suboval in outline. Owing to the absence of any dark pigment in the integument or gut they are difficult to detect against a white or pale background. They react like the adults to heat, moisture and light, except that they die more readily when floating on the surface of water.

No further development occurs unless the protonymphs obtain a full blood-meal, and the majority, if unfed, die at 25° C. on the fifth to sixth day.

Feeding (Tables III and IV). It is convenient to state at this point that two types of gorged protonymph can be distinguished by size. The smaller type (0.5 mm. long by 0.27 mm. broad) (Plate VIII, fig. 6) is destined to moult eventually into an adult male, whilst the larger type (0.7 mm. long by 0.34 mm. broad) (Plate VIII, fig. 5) will moult to become the adult female. There is no apparent method of distinguishing the sexes before the nymphs have matured except by size, so that in sexing nymphs a few errors arise owing to the occurrence of small females. The gorged nymphs are designated in the table as ♂N (male nymphs) and ♀N (female nymphs).

Both types are elongate, broadly ovoid in form, the body showing densely red or reddish-brown, while the legs and the capitulum and the area immediately behind it are semi-transparent and ochreous in appearance. Nymphs which have been engorged for several hours show a fine white Y-mark on the dorsum and a white anal area ventrally.

In the early part of this work attempts to feed protonymphs on naked young rats confined in boiling-tubes or by the scarified rat-tail technique proved unsatisfactory as a means of obtaining fully fed nymphs. In a large batch of protonymphs only a few would settle to feed, and they imbibed only a partial meal, even over a period of several hours. In one instance six protonymphs were induced to feed on a rat's tail on two separate

occasions, but both meals were partial and were completely digested, and no moult occurred. The nymphs proved more sensitive to disturbance than the adults, and, in view of the difficulty of confining such small stages in the containers in use, the methods were abandoned.

The only practical method of obtaining complete engorgement of protonymphs has been described on p. 233.

The results of an experiment using this technique are shown in Table III. The mites were allowed two hosts upon which to feed, and were given free access to the first

TABLE III

Showing the time required for *L. bacoti* protonymphs to attain full engorgement when free to feed at will on cotton rats. About 200 protonymphs were used, and the period during which each cotton rat was available was as follows

Host	Period on saw-dust after commencement of experiment	Period over water after commencement of experiment	Total period as host in experiment
1st	1st-18th hour	19th-72nd hour	1st-72nd hour
2nd	20th-44th hour	45th-72nd hour	20th-72nd hour

Mites recovered from hosts

Host	Hours after commencement of experiment	Protonymphs recovered from hosts		
		Gorged ♀ N	Gorged ♂ N	Total gorged nymphs
1st	18th*	3	13	16
"	19th	2	3	5
"	20th	1	4	5
"	21st	—	3	3
"	22nd	—	—	—
"	24th	1	6	7
"	28th	—	—	—
"	30th	1	2	3
"	31st	1	2	3
"	43rd	20	—	20
2nd	43rd*	—	2	2
1st	44th	3	—	3
"	45th	1	—	1
2nd	45th	1	—	1
1st	46th	3	—	3
"	48th	8	—	8
"	49th	3	—	3
"	50th	2	—	2
"	66th	—	1	1
"	71st	1	—	1
Total mites from 1st host		50	34	84
Total mites from 2nd host		1	2	3
Total mites from both hosts		51	36	87

* Indicates collection from the sawdust under the host.

host on a sawdust-tray for a period of 18 hours before the rat was placed over water. At this point the sawdust was spread on filter-paper, and after all gorged mites had been removed the sawdust was placed under the second host. The second host remained 24 hours on the sawdust before being placed over water. It was considered that nymphs, particularly if unfed, might be overlooked in the sawdust, and the second host was used to provide the necessary blood-meal. It was also our intention to transfer to this second animal partially gorged or unfed nymphs falling from the first host, but in fact only gorged nymphs were recovered from the first host.

Table III shows that the majority of the male nymphs engorge and leave the rat within 24 hours, but that, although a few female nymphs are replete in 18 hours, most of them do not drop engorged from the host until between 30 and 50 hours after coming into contact with the host.

The recovery-rate of 45 per cent. of the nymphs released is less satisfactory than the recoveries for adults (Table I). This series of nymphs was five days old when the experiment began, and deaths were occurring in the batch. Had they been used one or two days earlier a greater percentage would probably have been recovered.

The direct observations on nymphs feeding on a rat's tail, and the fact that we have noted them feeding spasmodically at an oozing natural scar on a naked young rat, suggest that the long period during which the nymphs remain on the host is spent partly free in the fur and partly in feeding periodically until engorgement is complete. Further evidence to support this view was derived from a cotton rat which died during the course of an experiment between the 10th and 23rd hour after some 370 protonymphs had been placed on it. The nymphs were given, in the first place, only three hours in which to become established on the host, but when the rat was placed over water large numbers of partially fed nymphs were recovered. The rat was therefore returned to the sawdust, where it was found dead on the following morning.

TABLE IV

Showing that protonymphs do not become fully engorged even after at least nine hours of being free to feed at will on a cotton rat, and that partially fed nymphs may remain on a dead cotton rat for at least one day. About 370 unfed protonymphs were released at the beginning of the experiment

State of host	Periods on sawdust after commencement of experiment	Periods over water after commencement of experiment	No. of live nymphs recovered	
			Gorged	Partially gorged
Alive	1st-3rd hour	—	Nil	Nil
Alive	—	4th-6th hour	1 ♂ N	44
Alive Died between	7th-9th hour 10th-23rd hour	— —	Nil	30
Dead	—	24th-27th hour	Nil	54
Dead	—	28th hour	Nil	16
Dead	—	29th-49th hour	Nil	31

Total partially fed nymphs recovered from host : 175

Table IV shows that 175 partially gorged nymphs were recovered from the host after at least nine hours, during which they could have engorged. It is possible that the dying rat was an unsuitable host in the nine hours before death, but this is unlikely, since, of approximately 55 females released at the same time, 42 gorged and nine partially gorged specimens were recovered. Partially gorged nymphs set aside at 25° C. did not survive more than seven days.

It appears, therefore, that protonymphs generally require at least 10 hours to become engorged on a cotton rat, and that during this period they may leave the host on one or more occasions in a partially fed state. Protonymphs may remain on a dead animal for as long as 26 hours.

Digestion and Moulting. The gorged protonymph exhibits little sign of digesting the meal, apart from the formation of the white dorsal Y-mark and the anal spot, and a darkening of the colour to a brownish-red tinge. Excrement is limited to a few small white drops. The mites remain quite active for several hours but eventually come to rest in order to moult. The interval between engorging and moulting is probably about 24 hours, but a precise figure is difficult to determine, since it is not always certain how long a nymph will remain at large on the fur in the engorged state. It is known certainly from one experiment that 10 female nymphs and 15 male nymphs free to gorge for 22 hours on a cotton rat moulted within 24 hours of leaving the host.

The cast skin from a gorged protonymph appears to be identical with that of the unfed protonymph, apart from an increase in size which is attained by the stretching of the body-integument between the various plates. The death observed in partially fed nymphs and the absence of cast skins on the fur of the host show that there is only one active blood-sucking nymph which feeds on the host and drops off to moult into the second nymph or deutonymph.

Shelmire and Dove (1931) state that newly hatched mites remain attached to the skin of the host (rats and mice) for approximately two days. It is true that they remain on the host for such periods, but it is apparent that for much of the time they are free in the fur and attached to the skin only for feeding periodically. We have not seen the nymphs feeding, as the above authors describe, on the eye-lids of cotton rats or of white rats, but we have seen adults and nymphs, in various stages of distension, swarming, after 24 hours without a host, into the fur of both types of rat, and the mites were visible moving in the fur of white rats for three days.

*The Moul*t (Plate VIII, fig. 7). Rupture of the protonymphal integument occurs along a line running from below one spiracle forwards above the base of the legs, round between the capitulum and the dorsal shield, and back along the other side to the other spiracle.

The deutonymph escapes by pushing backwards and upwards, a movement which results in the dorsum of the skin being flexed backwards like a lid—so much so that it becomes forced back and beneath the deutonymph, and the skin becomes compressed and folded in the region of the posterior dorsal plate and again near the anus. The capitulum and legs are last to be moulted.

The actual process of moulting is rather slow and laboured, and about two or three nymphs in a batch of 150 nymphs or so die in the process.

THE DEUTONYMPH (Plate VIII, fig. 8)

This stage follows the protonymph, and there are two sizes of deutonymph, corresponding to the gorged male and gorged female protonymph. There is little or no change in the dimension of the nymphs after the ecdysis; slight digestion of the protonymph's blood-meal is indicated by a darkening of the distended gut diverticulæ to a purple-red colour, and an intensification of the whiteness of the Y-mark and the anal spot. We have not seen excrement being discharged in this stage.

Although the distribution of hairs and plates in the deutonymph is similar to that of the protonymph, the integument of the deutonymph lacks the ochreous tinge seen in protonymphs. The capitulum and appendages of the living deutonymph have a glistening, semi-transparent and rather glabrous appearance. The palps are, in life, held closely against the central rostrum and give the capitulum the appearance of being a semi-transparent shiny cone. In mounted preparations the palps are found not to be fused with the rostrum, although this is the impression gained from living specimens. Details of the chelicerae have not been fully investigated, but it has been noted that the digits are small and lightly chitinized.

Deutonymphs do not feed, and, like the larva, they are relatively inactive, although warming a storage-tube by hand or particularly with a bench-lamp will induce rapid running movements.

Within 24–36 hours the deutonymphs moult into adult males or females according to size, and, as in the previous ecdysis, a few may die during the moult (seven in 150).

The method of moulting from the deutonymph is similar to that already described for the protonymph. The cast skins of the protonymph and the deutonymph can be readily differentiated by the absence of pigmentation and by the transparency of the integument in the latter.

Deutonymphs have been found only in samples of bedding-material, and, since they are not taken in water placed beneath rats and cast skins are not found in the fur, it is clear that protonymphs when engorged drop from the host and that the deutonymph stage occurs off the host.

Shelmire and Dove (1931) state that four or five blood-meals and three or four moults are required before the larva (by which they imply the young stage in a general sense) reaches maturity. Dove and Shelmire (1932) state that at least four blood-meals are required for the life-cycle, and that under 'favorable conditions' engorgement of 'different stages of mites' occurs at intervals of about three days. We agree that several partial blood-meals may be taken at intervals by the protonymph, but we are satisfied that only one complete engorgement is necessary for this stage to become adult, and that two moults intervene between this engorgement and the adult.

THE EARLY ADULT PHASE

The freshly emerged adult has the same general dimensions and degree of distension as the deutonymph and protonymph from which it is derived, but digestion becomes accelerated and is accompanied by the excretion of faecal material in considerable quantities, the drops being at first mainly white and paste-like but later showing the black or dark-pigmented granules from undigested blood in the gut. The excreta are deposited at random. This digestive process is accompanied by a progressive flattening of the body;

the gut pattern becomes apparent and the colour of the gut changes to a lighter brown. The adults become increasingly active and characteristically tend to push into the cotton-wool plugs in storage-tubes. Death follows without oviposition in 7-10 days in the absence of a blood-meal. Mating occurs certainly on the second day of adult life, and a blood-meal is readily taken as early as the second to third day after emergence.

SUMMARY OF THE LIFE-CYCLE OF *L. bacoti*

In the following time-table the feeding phase of the mites was spent on a cotton rat in a moderately dry atmosphere in a well-lighted laboratory at 21-22° C., and at other times the mites were stored in an incubator in dry tubes at 25° C.

Adults emerge, mate, and engorge within	3	days
Earliest eggs laid within	2	"
Larvae emerge within	1½	"
Larvae moult to protonymphs within	1	"
Protonymphs harden, feed, and drop from host within	2	"
Gorged protonymphs moult to deutonymphs within	1	"
Deutonymph moults to adult within	1	"
Total time required for one complete cycle	11½	"

This estimate for the total life-cycle is in agreement with the observations of Dove and Shelmire (1932), who record that under 'favorable conditions' the life-cycle is completed in as short a time as 12 days.

THE DEVELOPMENT OF *L. carinii* IN *L. BACOTI*

The account given here of the development of *L. carinii* in the mite is based on data from (i) dissections of gorged females taken at random from the bedding of infected cotton rats and (ii) dissections of gorged nymphs and females given a single infective blood-meal on an infected cotton rat on a known date.

* Two experimentally infected series of mites have been examined. In one series the unfed protonymphs were engorged on an infected cotton rat by the technique described on p. 233, and, using the same technique in the second series, females were given only the first adult meal on an infected cotton rat. In both series subsequent meals were given on uninfected white rats, using the scarified tail technique (p. 234). Thus, in each series, the time of the initial infection is known within limits varying from one hour to about 48 hours. Neither series was large enough to permit of a systematic dissection of a definite number of mites at fixed intervals. Dissections have been made chiefly to assess the intake of microfilariae and to obtain a guide as to the percentage of mites becoming infective and the time required for the microfilariae to reach the infective stage. Intermediate stages were looked for in such mites as died or were found dying during the period of the experiment. Examinations of freshly made dissections for microfilariae were checked by subsequent staining with Leishman's stain to show sheaths.

The cotton rat hosts in both experiments showed a concentration of from 1,900 to 2,200 microfilariae per c.mm. of peripheral blood taken from the thigh, as estimated against white blood-corpuscles.

Microfilariae. Eighteen per cent. of 49 gorged females collected at random and containing unaltered blood-corpuscles showed active sheathed microfilariae. The number of such microfilariae seen in a mite varied from one to four, and in one instance the microfilaria was found at least 24 hours after the last blood-meal had been taken.

In the experimentally infected series of protonymphs 25 mites dissected within 24 hours of the infective blood-meal revealed no active microfilariae, despite the ingestion of an average of 45 microfilariae per male nymph (range 15–70) and 91 microfilariae per female nymph (range 12–223), as estimated from empty sheaths in the stained film. In one of the female nymphs a microfilaria was seen in its sheath in the stained preparation. This microfilaria may have been ingested from about 10 minutes up to 46 hours before the dissection. Unfortunately, in the experiment on infection at the adult stage, only stained slides were examined; but empty sheaths counted in dissections of 20 females within from one to three hours after the meal varied from five to 289 (average 61) per mite.

The size of the microfilariae, excluding the sheath, at the time of ingestion varies from 90.2μ to 103.4μ .

Intermediate Developmental Forms (lengths up to 300μ). In a series of dissections of 154 females taken at random from infected rats, 9 per cent. showed intermediate developmental forms ranging from 115μ to 300μ in length. In 12 mites in which the number of worms was counted, a maximum of three per mite was noted. In the case of one mite which had been starved for five days a developmental stage 115μ by 6.6μ was obtained. In the two experimentally infected series a total of 31 females were dissected at intervals of from one to 17 days after the infective meal. Except for one parasite about 130μ in length found on the seventh day in a female infected in the first adult meal, no developmental stages were found.

The mites were teased apart in the dissections, and it was not possible to determine the site in which development had taken place.

Late Developmental Forms (active worms exceeding 300μ in length). In fresh preparations of a series of 400 random females only 0.25 per cent. were positive, one mite showing an active form 506μ in length. This particular mite, kept at $18-20^{\circ}\text{C}$. for five and a half hours after its last blood-meal, contained also two active microfilariae and two later forms about 161μ in length.

Eighteen females, exposed to infection at the nymph stage, were dissected from 20 to 30 days after the infective meal. All were found negative. Thus, no developmental stages were seen in mites infected as nymphs.

In the series of mites exposed to infection at the first adult meal, 11 were dissected from 20 to 33 days later, and of these five were positive (45.5 per cent.). Three of the mites dissected on the 20th day contained worms from $420\mu^*$ to $472\mu^*$, one mite showing two parasites and the others only one. A female dissected on the 25th day was found to have five parasites, and, although four had been damaged in the dissection, one of these must have been at least $660\mu^*$ in length. On the 33rd day one mite contained one active worm 935μ in length in which a tubular gut was visible. This would appear to be the infective form which is given by Williams and Brown (1946) as being between 800μ and $1,000\mu$ in length.

These late developmental forms, presumably pre-infective and infective forms, were not intimately associated with the musculature of the capitulum and appeared to be free in the haemocoel.

* As measured in dry films; other measurements from wet films.

Discussion. There is evidence to show that microfilariae are ingested in considerable, although variable, numbers by nymphs and adult mites, but that the majority fail to develop. No developmental stages have been observed in 24 mites exposed to infection as nymphs and dissected between two and 30 days after the infective meal, and it is possible that the slow digestion occurring in the deutonymph is unsuited to the development of the earlier stage of the parasite. On the other hand, six out of 36 females (16.7 per cent.) exposed to infection as adults were found positive in dissections from three to 33 days later. Of 11 females of the series dissected from 20 to 33 days later, five (45.5 per cent.) were positive for parasites from 420μ to 935μ in length.

It is noteworthy that none of the late developmental forms found by us were associated with the mouth-parts of the mite. If, as appears probable, these were infective forms, this observation suggests that transmission may not be directly associated with the bite of the mite.

TRANSMISSION

Although the cotton rat is the most suitable animal yet discovered for use in the chemotherapy of filariasis, it suffers from the disadvantage that it is not readily available in large numbers outside the countries of its origin. While colonies of cotton rats could be built up within a short space of time, it would be an obvious advantage to establish the filarial infection in a common laboratory animal such as the white rat. That the laboratory rat can function as a host of *Litomosoides carinii* has been shown by Chandler (1931), who recorded that a white rat which had been housed with cotton rats was also found to be infected. More recently, Williams and Brown (1946) have shown that white rats may be experimentally infected in the laboratory by exposing them to infective mites, microfilariae appearing in the blood of the white rats 80 days after exposure.

Our own endeavour, like that of other workers, has been to establish the infection in white rats, although uninfected cotton rats have also been used in order to compare the difference in the degree of infestation with *Litomosoides* resulting in each type of animal.

At the time of writing 45 white rats and 19 cotton rats have been exposed to infection by infective mites. Four of these white rats were exposed to infection at an early stage in the investigation. Of these, two rats, kept in separate cages, were housed with an infected cotton rat in a fibre-board box, in which sawdust served as a breeding-ground for the mites. After 59 days the rats were removed and their blood was examined at intervals until the time of autopsy, 97 days after their first exposure to infection. The remaining two white rats were infested with 200 potentially infective adult *L. bacoti*. The blood of these rats also was examined periodically for microfilariae up to the time of autopsy, 82 days after the first exposure to infection.

The greatest number of white rats, however, were exposed in the course of a single experiment, in which white rats and uninfected cotton rats were housed in close proximity to infected cotton rats. At the start of the experiment the community consisted of 10 white rats, three uninfected cotton rats and five infected cotton rats, the white rats being housed in two groups of five each in 1 ft. cubic wire cages, the cotton rats in separate cages half this size and each containing one rat. The rats were kept on a large wooden tray, as described earlier in this paper, on a bedding consisting of sawdust covered over with hay and cotton fibre. The mites which were added to the community had been maintained solely on infected cotton rats for a period of three months, and were accordingly potentially

infective at the start of the experiment, which was allowed to run for 31 days. In order to increase the risk of infection to the white rats, and because the mites showed some selectivity in favour of the cotton rats, the cotton fibre from the infected cotton rat cages was interchanged with that from the white rat cages on the 15th day of the experiment.

Of the 10 white rats originally added, nine were exposed for 31 days and one for 22 days. This latter rat gave birth to a litter of six 22 days after the start of the experiment, and was removed from the community 24 hours later. A further two white rats produced litters, totalling 11 in all on the 30th day, and these, together with the young rats, were removed from the colony after a 24-hour exposure to infective mites.

Of the uninfected cotton rats all three survived for 31 days. In addition, one of the infected cotton rats produced a litter of five, which was allowed to remain in contact with infective mites for at least 24 hours prior to its removal from the community. In this experiment, therefore, 27 white rats consisting of 10 adult and 17 baby rats, and eight cotton rats, consisting of three adult and five baby rats, were exposed to infection.

In addition to these rats, a further eight white rats have been exposed to infection by placing them in single mite-infested units, previously occupied by infected cotton rats, and six rats have been used for feeding potentially infective mites. As regards cotton rats, two adult rats and a litter of three baby rats have been exposed to infection for periods varying from 24 to 30 hours, while an additional six rats have been exposed for a period of four days.

The results of the majority of these experiments are not yet available, but those so far obtained have been as follows. The two rats kept in a mite-infested community with a single cotton rat failed to show microfilariae in their blood, and no worms were found in them at autopsy. The same negative result was obtained from blood examinations, made at weekly intervals from the 68th to the 81st day, of the rats exposed to infection by means of 200 mites. By way of contrast, however, the post-mortem findings in these rats were so striking as to warrant discussion in some detail. Autopsy was performed 82 days after the first exposure to infection. On opening the thorax three mature female worms, one immature worm and three mature male worms were found free in the left pleural cavity. In addition to these, one mature worm was found dead and encapsulated and three worms partially encapsulated but still showing signs of movement. The pleura was covered with circumscribed deposits firmly adherent to the wall, the condition resembling a fibrinous pleuritis. There was an excess of fluid of a cloudy nature in the pleural cavity, which on examination was seen to contain a large number of leucocytes, together with microfilariae. Microscopic examination showed that many of the microfilariae were surrounded by groups of cells that adhered to the larval parasites, which in some cases appeared to have undergone degenerative changes. Examination of heart blood revealed motile microfilariae, but no microfilariae were found in the peripheral blood. It is pointed out that the worm parasites and the changes described above were confined to the left pleural cavity; the right pleural cavity, which contained no worms, showed no abnormality. In the case of the second rat of this group, autopsy revealed two worms, both in the pleural cavity and both dead, fragmented and encapsulated. The blood of this rat also had been examined weekly from the 68th to the 81st day, with negative results.

Results of the large-scale transmission-experiment, in so far as they are available, confirm those recorded above and are as follows. Of the rats originally exposed to infec-

tion, one cotton rat died on the 33 d day and one white rat on the 48th day. Immature worms were found in the pleural cavities of both these rats, five in the cotton rat and two in the white rat. Despite these results, however, which indicated that the infection was being readily transmitted, examination of the blood of the white rats from the 61st to the 74th day was negative for microfilariae. One cotton rat, however, first exposed to infection on the same date, showed microfilariae in the blood 70 days later. On the 74th day three white rats of this group were killed and examined. No worms were found in two of these rats, but the pleural cavity of the third contained two male worms and one female worm, which appeared to be quite normal. The pleura appeared normal, but an increased volume of fluid, cloudy in appearance, was present in the pleural cavity. This fluid contained microfilariae, some active, some sluggish, surrounded by leucocytes, and some in various stages of degeneration. The heart blood and peripheral blood were negative for microfilariae.

A cotton rat exposed for the same period, under the same conditions as these white rats and autopsied on the same date, showed several worms in the pleural cavity, all apparently normal. The pleura was normal, and, although there was an apparent increase in the cellular content of the pleural fluid, the microfilariae were much more active than those found in the pleural cavity of the white rats, and no leucocytes were found adhering to them.

We may conveniently summarize these results by saying that, although we have been able to infect white rats with *L. carinii* by exposing them to infective mites, microfilariae have never been observed in the peripheral blood of two rats up to the 81st day after first exposure to infection and of nine rats up to the 74th day. While it is conceded that examinations were only made at weekly intervals from about the 60th or 70th day onwards, it appears unlikely that microfilariae would have been missed had they been present in the blood for any length of time during this period. The post-mortem findings of microfilariae in the pleural cavity and the heart blood, but not in the peripheral circulation, would appear to suggest that the microfilariae had not yet reached the general circulation in appreciable numbers. It seems highly probable that, had the rats been allowed to survive, microfilariae would have appeared in the peripheral blood within a few days. How long they would have persisted, in view of the conditions present in the pleural cavities of the rats, is a matter for speculation. The fragmented and partially or wholly encapsulated worms seen in the white rats have never been observed by us in cotton rats which have been naturally infected, and it would appear that some difference exists in the host-parasite relationship in the two types of animals.

Apart from these results obtained from white rats exposed to infection by infective mites, somewhat similar findings have been made in white rats to which adult *L. carinii* were transferred by surgical means. In the majority of cases the worms were transferred to the pleural cavity, but a few were placed in the peritoneal cavity. The blood of rats to which worms had been transferred was examined daily. Microfilariae in small numbers appeared in the peripheral blood from 14 to 18 days after the time of transfer, and in two cases they were still persisting when autopsy was performed 22-23 days later. Post-mortem examinations of these rats, made either at the first appearance of microfilariae in the blood or after these had been present for several days, showed that the adult worms in every instance were dead and encapsulated. Unfortunately it has not so far been possible to repeat these experiments with cotton rats, the cotton rats always dying during the course of the operation.

Although sufficient experimental data upon which to base a statement is not yet available, the results so far obtained would appear to suggest that, until further work has been done, caution should be exercised in accepting the white rat as a suitable host of *L. carinii*. The mere appearance of microfilariae in the peripheral blood cannot be accepted as evidence that the infection has been established in white rats.

SUMMARY AND CONCLUSIONS

1. The authors discuss the conditions required in a strain of filariasis suitable for chemotherapeutic research.

2. The present paper is a report of their investigations on the transmission of the filaria worm *Litomosoides carinii* to cotton and white rats by the mite *Liponyssus bacoti*.

3. Their techniques for the transmission of the infection and for the maintenance of the vector are described and discussed.

4. The life-cycle and habits of *L. bacoti* are described, with special reference to its maintenance and manipulation in experimentation. The life-cycle consists of the following stages: the adult male and female; the egg; the non-feeding larva; the blood-sucking protonymph; and the non-feeding deutonymph. Two engorgements, one by the protonymph and one by the adult, are required to complete the life-cycle, which at 25° C. takes from 10 to 12 days. Unfertilized females reproduce parthenogenetically. Adult females have been kept alive for 33 days on six engorgements, eggs up to a maximum of 12 being laid between meals. Unfed protonymphs survived up to six days, and unfed males and females lived for 11 and 10 days respectively.

5. Microfilariae are taken up in considerable, although variable, numbers by nymphs and adults of *L. bacoti*. Only a few microfilariae develop to later stages. No developmental forms were found in mites exposed to infection as nymphs, but, in females exposed to infection in the first adult meal, 45.5 per cent. of 11 mites showed from one to five parasites 420 μ –935 μ in length in 20–33 days after the infective meal. A parasite 935 μ in length, presumed to be an infective form, developed between 25 and 33 days after the infective meal. The method of transmission to the rodent host has not been observed.

6. *L. carinii* has been transmitted to cotton rats and to white rats as a result of exposure to infective *L. bacoti*.*

7. Post-mortem examination of white rats first exposed to infection 74 and 82 days previously revealed adult worms and microfilariae in the pleural cavity and microfilariae in the heart blood, but these were not found in the peripheral circulation at the time of autopsy.

8. Dead, fragmented and encapsulated worms and marked cellular reaction against the microfilariae were observed in white rats exposed to infection by infective mites. Such changes were not observed in one cotton rat exposed to infection for the same period and under the same conditions as the white rats, nor have they been observed in cotton rats naturally infected with *L. carinii*.

9. *L. carinii* has been surgically transferred to the pleural cavities of white rats. Microfilariae appeared in the blood 14–18 days after transfer, and in some cases persisted until the time of autopsy 22–23 days later. Post-mortem examination made either at the

* Since the above was written, in addition to the results recorded on p. 249 *et seq.*, two cotton rats, exposed to infection immediately after birth for four and nine days respectively, have shown microfilariae in the peripheral blood 71–75 days subsequent to exposure.

first appearance of the microfilariae or after they had persisted for several days showed the adult worms to be encapsulated, dead and fragmented.

10. Although the bulk of the results are not yet available, it is suggested on the basis of those so far obtained that caution be observed in accepting the white rat as a satisfactory host of *L. carinii* for chemotherapeutic research.

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EXPLANATION OF PLATE VIII

FIG. 1. Hatched eggs: natural, unmounted.

FIG. 2. Larva: mounted in polyvinyl-lacto-phenol.

FIG. 3. Larval moult: natural, unmounted.

FIG. 4. Protonymph: unfed; mounted in polyvinyl-lacto-phenol.

FIG. 5. Protonymph: gorged female type; cleared in NaOH and mounted in polyvinyl-lacto-phenol.

FIG. 6. Protonymph: gorged male type; cleared in NaOH and mounted in polyvinyl-lacto-phenol.

FIG. 7. Moult of gorged protonymph: mounted in polyvinyl-lacto-phenol.

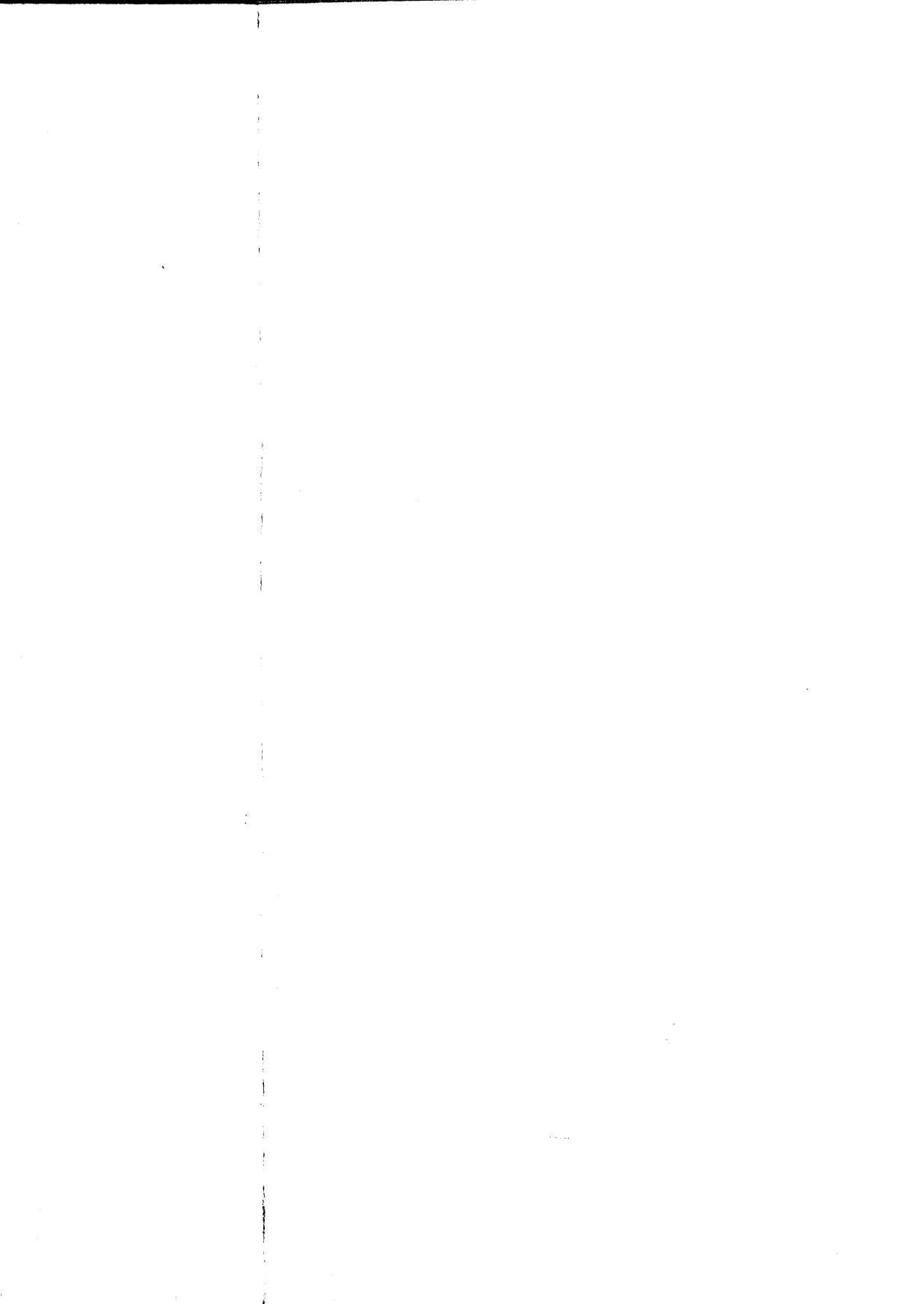
FIG. 8. Moult of deutonymph: mounted in polyvinyl-lacto-phenol.

FIG. 9. Male, showing some distension: cleared in NaOH and mounted in polyvinyl-lacto-phenol.

FIG. 10. Female, showing gut pattern at complete digestion: mounted, in polyvinyl-lacto-phenol.

FIG. 11. Female, partially gorged: cleared in NaOH and mounted in polyvinyl-lacto-phenol.

FIG. 12. Excreta droplets of female: natural, unmounted.



INTERFERENCE WITH THE TRYPANOCIDAL ACTION OF γ -(*p*-ARSENOSOPHENYL)BUTYRIC ACID BY *p*-AMINOBENZOIC ACID

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A number of examples are now known of the antagonistic effect of one substance on the chemotherapeutic activity of another. An early illustration of this was provided by Browning and Gulbransen (1922), who showed that parafuchsin can interfere with the therapeutic action of acriflavine in trypanosome infections, and it was later found that parafuchsin can interfere also with other trypanocidal agents, such as arsacetin or arspenamine (Schnitzer and Rosenberg, 1926). Attempts were made to interpret this particular series of interference-effects in terms of Ehrlich's receptor theories, but their explanation has remained obscure (Browning and Gulbransen, 1922, 1927; Schnitzer, 1926; Schnitzer and Rosenberg, 1926).

Other and later examples of chemotherapeutic interference were more readily interpreted. Thus, Voegtlin, Dyer and Leonard (1923) showed that various sulphhydryl compounds, glutathione in particular, antagonize the trypanocidal activity of a representative arsenoxide, *m*-amino-*p*-hydroxyphenylarsenoxide. This was explained by Voegtlin and his colleagues on the basis that the trypanocidal action of such an arsenoxide is due to a specific and lethal reaction between this type of compound and essential sulphhydryl components of the trypanosome cell. If, therefore, an excess of sulphhydryl-containing substances be supplied, there is a greater chance that the arsenoxide, by combining with part of this excess, will be deflected from the essential sulphhydryl components of the trypanosome cell. Among other instances of such interference with chemotherapeutic activity, the example which is probably best known, and which is of most far-reaching significance in the recent development of theories of the mechanism of drug-action, is the reversal of sulphanilamide-activity by *p*-aminobenzoic acid (Woods, 1940). Subsequent work by many investigators has fully borne out Woods' interpretation that this interference-effect is due to the fact that *p*-aminobenzoic acid is a necessary factor for the nutrition of the bacteria concerned; the antibacterial properties of sulphanilamide, structurally similar to *p*-aminobenzoic acid, are regarded as attributable to competition between these two substances for an enzyme necessary for the utilization of *p*-aminobenzoic acid by the

* With a grant from the Medical Research Council.

bacterial cell; and excess of *p*-aminobenzoic acid therefore favours the nutrition of the cell rather than its damage by sulphanilamide.

Another property of *p*-aminobenzoic acid was demonstrated by Sandground and Hamilton (1943*a*, 1943*b*). They showed that this substance can significantly reduce the toxicity to rats of aromatic pentavalent arsenicals, such as tryparsamide, carbarsone, etc., and of the trivalent neoarsphenamine, though not of certain other trivalent arsenicals, notably arsphenamine and 'mapharside' (*m*-amino-*p*-hydroxyphenylarsenoxide, OAs. $C_6H_3(NH_2)OH$). It is of particular significance for the work here to be reported that Sandground and Hamilton found, however, that *p*-aminobenzoic acid exercises no antagonistic effect whatever on the *trypanocidal* activity of any of the arsenicals named.

Reports have recently appeared of the *trypanocidal* activity of a new arsenoxide preparation, γ -(*p*-arsenosophenyl)butyric acid, OAs. $C_6H_4(CH_2)_3COOH$ (Eagle *et al.*, 1944; Eagle, 1945*a*). A special feature of this compound is that it is effective against trypanosome strains which have become resistant to tryparsamide and other commonly used arsenicals. We have found that its *trypanocidal* activity against both normal and tryparsamide-resistant trypanosomes can be antagonized by *p*-aminobenzoic acid, and this is described and discussed below.*

INTERFERENCE BY *p*-AMINO BENZOIC ACID *IN VIVO*

As a preliminary, we confirmed Eagle's claim (1945*a*) that the arsenosophenylbutyric acid under consideration is effective against trypanosomes which have been made resistant to tryparsamide. Our resistant strain of *T. rhodesiense* has retained its tryparsamide-fastness unimpaired since this character was produced 17 years ago (Yorke and Murgatroyd, 1930). Groups of mice infected respectively with the normal and with the tryparsamide-resistant *T. rhodesiense* were treated intraperitoneally with arsenosophenylbutyric acid two days after inoculation, when there were about 1 to 20 parasites per microscope-field. The ED₉₀ (i.e., dose causing temporary or permanent disappearance of trypanosomes from the blood in 90 per cent. of mice) was found to be about 0.015 mgm. for both the normal and the tryparsamide-fast strain.

Table I shows the results of a number of experiments in which infected mice were treated by the ED₉₀ of arsenosophenylbutyric acid, with and without the prior injection, also intraperitoneally, of a dose of *p*-aminobenzoic acid. In those cases where both substances were administered, the arsenical followed immediately after the *p*-aminobenzoic acid, the latter being given in the form of the sodium salt, previously prepared by neutralizing with the appropriate amount of sodium hydroxide, and then diluting with distilled water so that the required dose be given in a volume of 0.5 c.cm. per 20 gm. body-weight. It will be seen that the therapeutic efficacy of the arsenical, in the dose under investigation, was practically eliminated by a prior injection of 5 mgm. or more of *p*-aminobenzoic acid, and that this result was observed equally in mice infected with the normal and in those with tryparsamide-resistant trypanosomes.

* The γ -(*p*-arsenosophenyl)butyric acid was kindly supplied by Dr. A. J. Ewins, F.R.S., of Messrs. May and Baker Limited. It was in the form of the hydrate, and solutions were prepared for injection by neutralizing with dilute sodium bicarbonate. Doses stated throughout this paper are in terms of the unhydrated oxide, and were given in a volume of 0.5 c.cm. per 20 gm. body-weight.

TABLE I

Showing interference-effect of *p*-aminobenzoic acid upon therapeutic activity of γ -(*p*-arsenosophenyl)-butyric acid in *T. rhodesiense* infections of mice

Prior dose of <i>p</i> -am. benz. acid mgm./20 gm. i.p.	No. of mice					
	Normal <i>T. rhodesiense</i>		Tryparsamide-resistant <i>T. rhodesiense</i>		Totals	
	Treated ars. phen. but. acid*	Blood cleared of trypanosomes	Treated ars. phen. but. acid*	Blood cleared of trypanosomes	Treated ars. phen. but. acid*	Blood cleared of trypanosomes
None	16	16	9	9	25	25 (100%)
0.05	5	5			5	5 (100%)
0.25	5	5			5	5 (100%)
1.0	10	6			10	6 (60%)
5.0	10	1	5	0	15	1 (7%)
20.0	10	0	5	1	15	1 (7%)
80.0†	12	0	4	0	16	0 (0%)

* 0.015 mgm. per 20 gm., i.p.

† Approximately maximum tolerated dose.

Table II shows that against 'mapharside,' on the other hand, no antagonistic effect is exercised by *p*-aminobenzoic acid. This is in line with the finding of Sandground and Hamilton (1943a, 1943b) that *p*-aminobenzoic acid does not interfere with the trypanocidal activity of any of the commonly used arsenicals. The ED₉₀ of 'mapharside' for infections with our normal *T. rhodesiense* was found, like that of the arsenosophenyl-butyric acid compound, to be about 0.015 mgm. per 20 gm. mouse, and the efficacy of this dose was quite uninfluenced by prior injection of even maximal amounts (80 mgm.) of *p*-aminobenzoic acid. (Against the tryparsamide-resistant strain 'mapharside,' in common with most other trypanocidal arsenicals, is of course ineffective.)

TABLE II

Showing absence of interference-effect by *p*-aminobenzoic acid upon therapeutic activity of 'mapharside' (*m*-amino-*p*-hydroxyphenylarsenoxide) in *T. rhodesiense* infections of mice

Prior dose of <i>p</i> -am. benz. acid mgm./20 gm. i.p.	No. of mice	
	Treated 'mapharside'*	Blood cleared of trypanosomes
None	10	9
80†	10	10

* 0.015 mgm. per 20 gm., i.p.

† Approximately maximum tolerated dose.

An experiment was performed to determine the importance, for the interference-effect, of time-relationships of the injections of *p*-aminobenzoic acid and the arsenosophenyl-butyric acid compound. The main result is shown in Table III, from which it is seen that, even with maximal doses of *p*-aminobenzoic acid, interference by this substance scarcely

comes into play if it be injected *after* instead of *before* the arsenosphenylbutyric acid compound. Actually, there did appear to be some slight effect, not shown in the table, where the arsenoso compound was injected first, since trypanosomes reappeared again in the blood of most of the mice treated in this way very much sooner than in the case of control mice treated by the arsenoso compound alone. However, the importance of injection of *p*-aminobenzoic acid before and not after the arsenical, in order to obtain maximal interference, seems clear.

TABLE III

Showing influence of time-spacing of injections upon *p*-aminobenzoic acid interference with therapeutic activity of γ -(*p*-arsenosphenyl)butyric acid

First injection	Interval (minutes)	Second injection	No. of mice	
			Treated	Blood cleared of trypanosomes
<i>p</i> -aminobenzoic acid*	60	Arsenosphenyl-butyric acid†	4	0
	30		5	1
	15		5	1
	10		5	0
	5		5	0
	0		5	2
Arsenosphenyl-butyric acid†	5	<i>p</i> -aminobenzoic acid*	5	5
	10		5	5
	15		5	5
	—	None	5	5

* 80.0 mgm. per 20 gm., i.p.

† 0.015 mgm. per 20 gm., i.p.

INTERFERENCE BY *p*-AMINO BENZOIC ACID *IN VITRO*

Summarized below are a number of experiments designed to demonstrate interference with the trypanocidal activity of γ -(*p*-arsenosphenyl)butyric acid by *p*-aminobenzoic acid *in vitro*. The first attempts (paras. 1 and 2 below) were not successful, but experimental conditions were eventually found (para. 3) under which the effect could be shown.

1. The minimum lethal concentration of the arsenosphenylbutyric acid compound against our normal strain of *T. rhodesiense* was found to be 1 in 100 million (tested in a medium consisting of equal parts normal saline and unheated rabbit serum, containing about 1,000 trypanosomes per c.mm., observed for 24 hours at 37° C.). In the presence, incorporated in the medium, of 1 in 4,000 *p*-aminobenzoic acid (close to the maximum tolerated by trypanosomes for 24 hours at 37° C.), or of 1 in 40,000, the trypanocidal titre of the arsenosphenylbutyric acid compound was found to be unaltered, i.e., 100 million.

2. The possibility was then considered that although *p*-aminobenzoic acid does not affect the minimum lethal concentration of arsenosphenylbutyric acid, as measured at the end of a 24-hour observation-period, it may significantly retard the *initial* rate at which trypanosomes are destroyed. Experiments were therefore performed to determine the trypanocidal titre of the arsenosphenyl compound (again 1,000 trypanosomes per c.mm., and 37° C.) at short intervals, up to five hours, in the absence and in the presence of varying amounts of *p*-aminobenzoic acid, ranging in concentration from 1/62.5 to 1/4,000. The results of a number of such experiments are collected in Table IV, from which it is

seen that the titres were not significantly affected by the presence of any concentration of *p*-aminobenzoic acid at any period of observation from 15 minutes to five hours. The *p*-aminobenzoic acid therefore exercised no appreciable retarding influence on the rate at which trypanosomes were destroyed by the arsenical. This point may be made clearer by quoting from the table that at 15 minutes, for example, the titre in the absence of *p*-aminobenzoic acid was one million. In the presence of 1/250 *p*-aminobenzoic acid the titre was also one million, but it should have been considerably less if this substance were capable of much retarding the rate at which trypanosomes are destroyed by the arsenical. Such differences as are shown on the table, for each observation-period, are well within the range of experimental error.

TABLE IV

Showing trypanocidal titres of γ -(*p*-arsenosophenyl)butyric acid, at different short-term observation-periods, in the absence and in the presence of varying amounts of *p*-aminobenzoic acid

Concentration of <i>p</i> -aminobenzoic acid	Trypanocidal titre of γ -(<i>p</i> -arsenosophenyl)butyric acid, in millions (tubes set up with four-fold dilutions)						
	Minutes						
	15	30	45	60	120	240	300
None	1	4	4	4-16	16	16	64
1/62.5		1		4	16		64
1/250	1	1	4	4	16	16-64	
1/1,000		4		4			
1/4,000		4		4	16		16

3. Suitable conditions for demonstration of the interference-effect *in vitro* were eventually suggested by *a*, *b* and *c* below, which should be considered in relation to one another.

(a) It may be assumed that γ -(*p*-arsenosophenyl)butyric acid, like other aromatic arsenoxides, disappears from the circulating blood with extreme rapidity after injection. Thus Murgatroyd, Russell and Yorke (1934) found in rabbits that after intravenous administration of 10 mgm. per kgm. body-weight of reduced trypanamide thioglycollate (condensation product of the arsenoxide form of trypanamide and thioglycollic acid) the trypanocidal titre of the blood-serum fell in 2½ minutes from the expected figure of 32,000 to 4,000, and in half an hour to 64, reflecting a corresponding rate of elimination of the arsenical from the circulating blood during that time. We have found that after intraperitoneal injection of 0.015 mgm. per 20 gm. body-weight of γ -(*p*-arsenosophenyl)butyric acid into mice (with or without prior injection of *p*-aminobenzoic acid) the circulating blood lost its trypanocidal activity within half an hour.

(b) As is the case with other arsenoxides it is to be expected that γ -(*p*-arsenosophenyl)butyric acid is quickly taken up by the trypanosome cell as a preliminary to exercising its lethal effect. According to Eagle (1945*b*) this absorption is of very considerable degree and occurs with extreme rapidity, so that 10 minutes after contact with γ -(*p*-arsenosophenyl)butyric acid the arsenic concentration in the organisms may attain a level several hundred times that of the surrounding fluid. It seems probable that lethal amounts of arsenic become absorbed by the trypanosome cell in a matter of seconds.

(c) Table III above showed the importance of injecting the interfering dose of *p*-aminobenzoic acid *before* and not *after* the arsenosophenylbutyric acid. The rationale of this is implicit in *a* and *b* above. We assume the critical period for destruction of the trypanosomes to be very brief indeed, probably to be measured in seconds rather than minutes, immediately following injection of the arsenical. It is during this very short critical period alone that the arsenical is present in the circulating blood in relatively high concentration (*a* above), and, brief though the period be, trypanosomes are nevertheless capable during this time of absorbing sufficient of the arsenical for their eventual destruction (*b* above). If, then, the interference of *p*-aminobenzoic acid with the therapeutic action of γ -(*p*-arsenosophenyl)butyric acid be ascribed to a protection of the trypanosomes from the arsenical during this very short critical period, it immediately becomes clear why the *p*-aminobenzoic acid must already be in the circulating blood when the arsenoso compound is injected, i.e., why the *p*-aminobenzoic acid should be injected *before* and not *after* the arsenical, if maximal interference is to be obtained.

After a few orienting trials, the experiment below was accordingly performed. In the preliminary trials the interesting observation was made that if trypanosomes, suspended in equal parts serum and saline, be brought into contact with about 1 in 25 *p*-aminobenzoate, either at 37° C. or at room-temperature, they are immediately immobilized; this striking effect on the trypanosome's locomotor system is apparently reversible and innocuous, since active motility is restored, on reducing the concentration of *p*-aminobenzoic acid, by the further addition of an excess of serum. This reversible immobilization by *p*-aminobenzoic acid is a visible indication of the fact that the substance is capable of profoundly influencing the trypanosomes without permanently damaging them; and we may perhaps assume that this influence, possibly accompanied by absorption or adsorption of the substance to the trypanosome cell, involves also the power of limiting the entry of γ -(*p*-arsenosophenyl)-butyric acid into the cell.

Experiment. Trypanosomes were suspended, in a concentration of about 1,000 per c.mm., in a mixture containing 0.3 c.cm. fresh rabbit serum and 0.15 c.cm. of 1 in 6.25 of the sodium salt of *p*-aminobenzoic acid in a Kahn tube at 37° C. After 30 seconds γ -(*p*-arsenosophenyl)butyric acid was added in the amount of 0.15 c.cm. of a 1/50,000 solution (chosen as somewhat the order of concentration to be expected immediately after injection of 0.015 mgm. of the arsenical into a mouse), and the mixture was allowed to remain (being stirred meanwhile) for exactly 10 seconds at 37° C. The tube was then centrifuged sufficiently to throw down the trypanosomes, and the supernatant replaced by 2.0 c.cm. supporting medium (equal parts rabbit serum and normal saline). Operations were timed so that exactly 2½ minutes should elapse up to this stage from the time that the trypanosomes came into contact with the arsenical. The tube was again centrifuged, and the supernatant again discarded, being replaced this time by 0.6 c.cm. supporting medium. The tube was then immediately returned to the water-bath at 37° C., the entire operation since the moment of contact between trypanosomes and arsenical being exactly five minutes. The preparation was thenceforth kept at 37° C., a small drop being removed at intervals for trypanosome count.

For comparison the whole procedure was then repeated in the same way, with exactly the same timing, with the single difference that the initial mixture in which the trypanosomes were suspended consisted of 0.3 c.cm. serum and 0.15 c.cm. saline, instead of 0.3 c.cm. serum and 0.15 c.cm. *p*-aminobenzoic acid.

Table V shows the result of the above experiment. It may be seen that trypanosomes which had been in contact simultaneously with γ -(*p*-arsenosophenyl)butyric acid and *p*-aminobenzoic acid were still alive 24 hours later, whilst those which had been in contact with the arsenical alone were destroyed within six hours.

TABLE V

Showing trypanosome counts at intervals after exposure *in vitro* for 10 seconds to 1/200,000 γ -(*p*-arsenosophenyl)butyric acid (a) in the presence, and (b) in the absence of 1/25 *p*-aminobenzoic acid

Time since contact with arsenosophenyl-butyric acid	Trypanosome count per c.mm. after contact with arsenosophenylbutyric acid	
	(a) In presence of <i>p</i> -am. benz. acid	(b) In absence of <i>p</i> -am. benz. acid
0	860	840
1 hour	875	625
2 hours	875	530
3 "	810	470
4 "	870	6
5 "	840	6
6 "	780	0
7 "	935	—
8 "	990	—
9 "	875	—
24 "	25	—

This experiment was repeated a number of times, using the same concentrations of the compounds concerned, and, though the result was not always as unequivocal as in the example above, it was always found that trypanosomes withstood impact of the arsenical *in vitro* better in the presence than in the absence of *p*-aminobenzoic acid.

INTERFERENCE BY GLUTATHIONE *IN VIVO*

Reference has already been made at the beginning of this paper to the demonstration by Voegtlin, Dyer and Leonard (1923) of the interference of glutathione with the trypanocidal activity of another arsenoxide, *m*-amino-*p*-hydroxyphenylarsenoxide (i.e., the partial oxidation product of arsphenamine); and Moncorps and Bohnstedt (1934) and Strangeways (1937) showed that this glutathione-interference operates also against neoarsphenamine. On the basis of these and related observations, the highly plausible theory was developed that the trypanocidal action of this arsenoxide (and of neoarsphenamine after oxidation to the arsenoxide) is due to a specific lethal reaction with the sulphydryl groups of glutathione and possibly of other sulphydryl-containing compounds in the trypanosome cell.

In considering the relevance of *p*-aminobenzoate-interference to the mode of trypanocidal action of γ -(*p*-arsenosophenyl)butyric acid, we thought it advisable to find out if glutathione can inhibit the trypanocidal activity also of this particular arsenoxide. The following experiment was therefore performed.

Experiment. Four groups, of five mice each, on the second day of infection with *T. rhodesiense* (about 1 parasite per microscope-field) were treated respectively as follows (intraperitoneally; doses per 20 gm. body-weight):

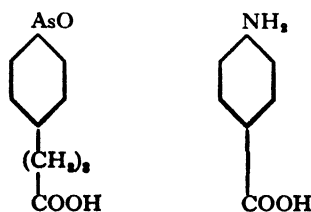
- (i) *m*-amino-*p*-hydroxyphenylarsenoxide, 0.02 mgm.
- (ii) As i, together with 30 mgm. glutathione.
- (iii) γ -(*p*-arsenosophenyl)butyric acid, 0.02 mgm.
- (iv) As iii, together with 30 mgm. glutathione.

The blood was then examined at half-hour intervals. It was found that in groups i and iii the blood was clear of trypanosomes within half an hour of treatment, but in groups ii and iv, which had received glutathione together with the arsenicals, it was 3-4 hours before trypanosomes were no longer to be seen. There was no apparent difference between these two groups (ii and iv) in respect of the time elapsing before the trypanosomes disappeared.

It may be concluded, therefore, that glutathione interferes equally with the therapeutic activity of *m*-amino-*p*-hydroxyphenylarsenoxide and of γ -(*p*-arsenosophenyl)-butyric acid.

DISCUSSION

It is tempting to seek an analogy between the interference with sulphonamide-activity by *p*-aminobenzoic acid, dependent on structural similarity between the two compounds concerned, and interference with the trypanocidal activity of γ -(*p*-arsenosophenyl)butyric acid by *p*-aminobenzoic acid, also on the basis of a (somewhat tenuous) structural resemblance, as below:



The only other substances, related to *p*-aminobenzoic acid, which we have examined for interference with the trypanocidal activity of arsenosophenylbutyric acid are *p*-hydroxy-

benzoic acid and benzoic acid, neither of which was found to exhibit this property. (Woods, 1940, also found that neither of these substances interferes with sulphonamide-activity.)

One of the most telling points of circumstantial evidence adduced by Woods (1940) in favour of the theory that *p*-aminobenzoic acid interferes with the bacteriostatic action of sulphanilamide by competition for the same enzyme, for which *p*-aminobenzoic acid is the substrate (or product), is the fact that, whilst there seemed to be no possibility of a direct molecular reaction between bacteriostatic agent (sulphanilamide) and antagonist (*p*-aminobenzoic acid), over a wide range the relative concentrations of these two substances required for exhibition of the interference-effect *in vitro* were nevertheless found to be constant. This ratio, sulphanilamide/*p*-aminobenzoic acid, was of the order of 10,000/1. In our *in vitro* experiment, described above, the inverse ratio was employed, i.e., arsenical/*p*-aminobenzoic acid approximately 1/10,000. We are not able to give any data to show whether this is a necessary ratio for demonstration of the interference-effect, over a wide range of concentrations, but, if we are to persist in seeking the above analogy, then we must surely hold that *p*-aminobenzoic acid is essential for the trypanosome's vitality, and that γ -(*p*-arsenosophenyl)butyric acid acts by preventing the trypanosome from utilizing this substance. There is, however, no evidence for this view. The fact that glutathione interferes with the trypanocidal action of γ -(*p*-arsenosophenyl)butyric acid equally with that of 'mapharside' strongly suggests that the essential mode of trypanocidal action of the arsenosophenylbutyric acid is similar to that of other arsenoxides, namely, by reacting fatally with sulphydryl-containing components of the trypanosome cell. It seems likely, therefore, that *p*-aminobenzoic acid exercises its antagonistic effect against the trypanocidal activity of γ -(*p*-arsenosophenyl)butyric acid by preventing the latter from reacting with sulphydryl-containing components of the trypanosome cell. The underlying mechanism by which this reaction is prevented remains obscure. It may be that both arsenosophenylbutyric acid and *p*-aminobenzoic acid are capable, simultaneously, of penetrating into the trypanosome cell, and that it is *in situ* that the arsenical is prevented from reacting with the sulphydryl components. It seems much more likely, however, that *p*-aminobenzoic acid in some way bars or limits the entry of arsenosophenylbutyric acid into the cell. In order to accept this we must also hold the corollary view that such exclusion of arsenosophenylbutyric acid from the trypanosome cell is a property which *p*-aminobenzoic acid exercises selectively against that particular arsenical, and not against 'mapharside' and other common arsenicals, with whose trypanocidal activity it does not interfere. It is easy to believe that the property in question may be exercised in this selective way, since there are very good grounds, as follows, for believing that the route of entry of γ -(*p*-arsenosophenyl)butyric acid into the trypanosome cell is fundamentally different from the route of entry of the commoner arsenoxides.

Unlike the commoner arsenicals, such as arspenamine and 'mapharside,' γ -(*p*-arsenosophenyl)butyric acid is active against both normal and tryparsamide-resistant trypanosomes. It does, in fact, belong to the group of aromatic arsenicals characterized by the possession of an acetic acid radicle, including arsenophenylglycine, for which compound Ehrlich (1909) entertained high hopes in the treatment of trypanosomiasis and syphilis. The members of this group are all active against trypanosomes which have been made resistant to atoxyl (*ergo* to tryparsamide), and Ehrlich (1909) endowed the trypanosome cell with 'aceticoceptors,' in order to explain the fact that atoxyl-resistant organisms, in which the 'arsenoceptors' were presumed to have lost their specific avidity, nevertheless

remained susceptible to this particular group of arsenicals. Especially relevant in this connection are the studies of King and Strangeways (1942) and King (1943). They pointed out that substituted phenylarsenoxides containing carboxyl groups (e.g., 4-carboxyphenylarsenoxide) exercise the same (though not a very high) degree of activity *in vitro* on trypanamide-resistant *T. rhodesiense* as on the normal strain. Clearly those arsenicals for which 'aceticoceptors' were postulated by Ehrlich fall within this category, as does also Eagle's γ -(*p*-arsenosophenyl)butyric acid, although the trypanocidal activity of the latter compound is exceptional for this class of arsenoxide. King and Strangeways argue that, by virtue of their solubilizing carboxyl groups, such compounds would form neutral sodium salts with a great affinity for water. These sodium salts are always present as ions, they would be loth to leave the watery medium (hence probably accounting for the relatively low trypanocidal activity of the group as a whole), and they would enter the trypanosome cell in the same way as other substances which are very soluble in water, such as glucose and salts. The other arsenoxides studied by King and Strangeways were, in general, much more trypanocidal and fell into two categories. One of these is represented by unsubstituted phenylarsenoxide, which Hawking (1937) found, like arsenoxides with carboxyl groups, to be equally active against trypanamide-resistant as against normal trypanosomes. These substances are devoid of hydrophilic groups, apart from the arsenoxide group, they are not ionizable, and it was suggested that the means by which they are taken up by the trypanosome cell, which consists largely of lipoid matter, is such that the phenyl (or in xylylarsenoxides the xylyl) group is in the lipoid and the arsenoxide group is at the water interface. The remaining category comprises substances—benzamide-*p*-arsenoxide, for example—which were 32 and 64 times more active against normal than against trypanamide-resistant trypanosomes, and these were pictured by King and Strangeways as being waylaid by adsorption on polar surfaces in normal trypanosomes *en route* to their site of action. King and Strangeways therefore envisaged three distinct types of distribution of aromatic arsenicals in or on the trypanosome cell, but they emphasized that these are merely primary phases, preparatory to the essential mechanism by which the trypanosome is destroyed; this eventual mechanism is a chemical one, dependent on lethal interaction between arsenoxide—once it has found its way into or on to the trypanosome cell—and sulphhydryl groups there encountered and necessary for the trypanosome's existence. Finally, in further support of the view that arsenoxides of the type of arsenosophenylbutyric acid differ from other types of arsenoxide in the means by which they enter the trypanosome cell, we may quote the finding of Eagle (1945*b*) that such penetration is dependent on *pH* in the case of acid-substituted arsenoxides (e.g., arsenosophenylbutyric acid) but not in the case of other types.

There appears, therefore, to be no serious obstacle to our view that *p*-aminobenzoic acid interferes with the trypanocidal action of γ -(*p*-arsenosophenyl)butyric acid not by influencing the interaction between arsenical and sulphhydryl groups in the cell, which we suppose to be the essential lethal mechanism (as it is in the case of other arsenoxides), but by selectively limiting the admission of this type of arsenoxide into or on to the trypanosome cell.

SUMMARY

It has been found that *p*-aminobenzoic acid interferes with the trypanocidal activity of γ -(*p*-arsenosophenyl)butyric acid, although it exercises no such effect against the more

usual type of arsenical, such as tryparsamide, arsphenamine and 'mapharside.' The interference-effect was demonstrated both *in vivo* and *in vitro*.

Glutathione also interferes with the trypanocidal activity of γ -(*p*-arsenosophenyl)-butyric acid, as it does with the activity of other arsenicals.

The view is expressed that, as is the case with other arsenoxides, the essential mode of trypanocidal action of γ -(*p*-arsenosophenyl)butyric acid is by reacting fatally with sulphhydryl-containing components of the trypanosome cell, and that *p*-aminobenzoic acid exercises its interference not by influencing this mechanism, but by selectively limiting the admission of this particular type of arsenoxide into or on to the trypanosome cell.

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THE INTRACUTANEOUS TEST IN CUTANEOUS LEISHMANIASIS

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In countries where leishmaniasis occurs endemically, the clinical diagnosis is, as a rule, determined by the characteristic aspect and parasitic findings, other diagnostic methods usually being superfluous. When reviewing a large series, however, one meets a number of cases simulating some trivial condition, e.g. those with impetiginous elements, as well as—especially in later stages—cases resembling lupus or syphilis, the clinical course being quite different from that of the classical type. In some cases of the classical type, as well as in deviating forms, parasitic findings are often negative. Failure to demonstrate leishmania bodies was reported in 25 per cent. of cases by Bettmann and Wasielewski (1909) and in 23 per cent. by Dostrovsky (1935). Particularly if there is a high degree of secondary infection, and if the lesions have been present for a long time and show the first signs of spontaneous healing, demonstration of the parasites in the lesion may be difficult. Occasionally it is possible, after a prolonged and thorough search, to detect a few parasites, but these, if they are not absolutely characteristic, do not justify a positive diagnosis.

Even more rare are positive parasitic findings in recurrent leishmaniasis, as in tubero-serpiginous, annular and similar varieties of the late type. Here the microscopical findings are only very exceptionally positive. In the last-mentioned cases it is because of the negative parasitic findings that they are erroneously spoken of as cases of 'lupus post leishmaniasis'; but in a considerable number of cases of nodular and especially of recurrent leishmaniasis culture results were positive even when repeated smears were negative (Dostrovsky and Sagher, *in the press*). The histological method is also of a certain, though more restricted, diagnostic value, leishmania bodies being sometimes demonstrable by this means when not seen in smears.

Nevertheless, there remain numerous cases in which a corroboration of the clinical diagnosis by an immune reaction is of the greatest importance. The first experiments with leishmania vaccine were, as a matter of fact, conducted for therapeutic purposes (Row, 1912; Jessner, 1927; Dostrovsky, 1929), while test-experiments were made by Montenegro (1926) with culture extracts. Dostrovsky (1935) demonstrated positive skin-reactions on intracutaneous injection of a leishmania vaccine containing 100,000 parasites per 0.1 c.cm. This reaction has now to be verified in a larger series of cases. It should, however, be pointed out at the outset that the value of this test has not yet been firmly established.

SPECIFICITY OF THE LEISHMANIA-VACCINE REACTION

During 1924-44 more than 600 cases of cutaneous leishmaniasis were treated at the dermatological clinic of the Rothschild-Hadassah University Hospital in Jerusalem. Smears for identification of parasites were made in 401 patients, 267 (66.6 per cent.) of which were positive and 134 (33.4 per cent.) negative.

The leishmania-vaccine reaction was performed in 261 leishmania patients, 239 of whom (91.6 per cent.) gave positive results and 22 (8.4 per cent.) negative results.

Table I shows results in cases where the cutaneous test and parasitic examination were both carried out.

TABLE I

Leishmania-vaccine reaction and parasitic findings in 217 cases of leishmaniasis nodosa

	No. of cases	Percentage
Leishmania-vaccine reaction positive ; parasitic findings positive	111	51.1
" " " negative ; " " positive	7	3.2
" " " positive ; " " negative	96	44.2
" " " negative ; " " negative	3	1.4
Total	217	100.0

From the above table it appears that the test has a high degree of specificity, since negative results did not occur in more than 4.6 per cent.

Even greater is the difference in recurrent leishmaniasis alone, in which parasites are only very exceptionally seen. The relation between the leishmania-vaccine reaction and parasitic findings in such cases is shown in Table II.

TABLE II

Leishmania-vaccine reaction and parasitic findings in 56 cases of recurrent leishmaniasis

	No. of cases	Percentage
Leishmania-vaccine reaction positive ; parasitic findings positive	3	5.4
" " " negative ; " " positive	1	1.8
" " " positive ; " " negative	43	76.8
No leishmania-vaccine reaction performed ; parasitic findings negative ...	4	7.1
Leishmania-vaccine reaction positive ; microscopical examination not performed	5	8.9
Total	56	100.0

From Table II it is seen that, among 51 cases examined, parasitic findings were positive in no more than four, and the method therefore cannot be regarded as a valuable help in diagnosis. On the other hand, the leishmania-vaccine reaction was positive in 51 out of 52 cases, i.e., in 98 per cent.

The probability of a non-specific reaction is low. Among 55 cases suffering from various dermatoses, Dostrovsky (1935) reported a slightly positive reaction in only 9 per cent., comprising five cases of mixed leprosy and one of psoriasis and sycosis. Moreover, no account had been taken of the country from which the patients came, and Doubrovskoy (1941) has emphasized that, in patients from countries where leishmaniasis occurs endemically, the test may be positive without any visible sign of an earlier leishmania infection. We also frequently found that in childhood leishmaniasis had completely escaped notice, and only a very thorough search produced an old scar somewhere on the body as an indica-

tion that the infection had been present. Particular difficulty arises when such a scar occurs on the skin of a patient suffering from leprosy, and in some of the above cases yielding a positive leishmania reaction this result may have been due to the fact that the patients came from countries where leishmaniasis occurs endemically. This is illustrated by our latest control-series in 94 patients with various dermatoses, in which special attention was paid to the patient's country of origin. Five of the patients yielded a markedly positive result, and two a slightly positive result, and it was found that, among the former, four hailed from Baghdad and one from Afghanistan, and presented old scars. The two remaining cases were Europeans who were suffering from furunculosis; here the reaction has indeed to be regarded as non-specific. The non-specificity in this series was, therefore, 2.2 per cent.

Since the patients visiting the clinic hailed from the most varying parts of the Near and Middle East, experiments with different leishmania strains were also set up. The majority of the patients, however, had acquired their infection at the Dead Sea or in Baghdad, and but very few in other parts of Palestine or in Iraq, Syria, Persia, Afghanistan, etc., and the vaccines were therefore prepared from cultures of lesions from the Dead Sea and Baghdad. Theoretically, there might be a possibility that the two types might show distinct differences in the skin-reactions produced by each.

TABLE III

	No. of cases
Palestinian strain and Baghdad strain equally positive	126
" " weaker than Baghdad strain ...	8
" " stronger " " ...	12
" " negative; Baghdad strain positive	1
Total	147

Table III shows that, essentially, there is no appreciable difference in the reactions of the vaccines prepared from the two strains. The insignificant quantitative deviations may very well be due to technical inaccuracy unavoidable with an injection of exactly 0.1 c.cm. Moreover, the differences showed no parallelism with the type of lesion presented by the particular patient. The one case in which the reaction with the Palestinian strain was positive while with the Baghdad strain it was negative is probably to be regarded as due to this source of error.

The sensitivity of the reaction is, therefore, independent of the type of parasite from which the vaccine had been prepared.

ONSET AND DURATION OF THE SKIN-REACTION

The skin-reaction can be elicited at a very early stage of the infection, and probably the mechanism of sensitization comes into being practically at the moment of inoculation. In order to determine how long after infection the skin-test becomes positive, leishmaniasis was artificially induced in three volunteers among the physicians of this clinic.

In one case, inoculation with 2,000,000 parasites was followed by the development of a leishmania lesion with practically no incubation-period. The intracutaneous reaction, originally negative, became positive after the test made on the second day after inoculation.

In the second case, also inoculated with 2,000,000 living parasites, the leishmania-vaccine reaction, which a fortnight earlier had been negative, was still negative on the

first day after inoculation, but on the 3rd and 13th days it was slightly positive, while after five weeks it was markedly positive.

In the third case, the reaction was positive two days after inoculation.

These few experiments show that the reaction very soon becomes positive—a feature which increases its clinical value.

The skin-sensitivity not only persists during the period of infection, but continues for years after the lesions have healed. This conclusion derives from cases in which an infection has undoubtedly occurred in childhood, the patients presenting a positive skin-reaction as late as 30–40 years afterwards. Practically, therefore, the skin-reaction may be regarded as remaining positive throughout life. In any event, attention should be paid to this possibility in countries where leishmaniasis occurs endemically.

SENSITIVITY OF THE TEST

A high sensitivity of the test presumably explains differences in the degree of reaction observed in different types of leishmaniasis. Thus, one of us (F. S.), in studies on the response to graded intracutaneous doses of vaccine, showed that the fresh nodular types most frequently give a positive reaction to dilutions of the vaccine of 1 : 100 to 1 : 10,000. Recurrences and early forms with lymphogenic spread show a much higher average level, the maximum lying between 1 : 10,000 and 1 : 10,000,000.

Although the reaction shows a high degree of constancy, nevertheless an occasional case of leishmaniasis may, to begin with, give a negative reaction, which only during the course of treatment gradually develops into a positive one. We saw this in one case only, as follows. The patient, a boy, presented a lesion which occupied almost the whole of his left cheek and extended up to 2 cm. in front of the ear and just beneath the eyelid. The lesion was reddish-brown, with a rusty tinge similar to that of leprosy infiltrations. Pressure with a glass spatula produced yellowish nodules in the depth of the skin. Several millimetres from the edge of the main lesion there were several fresh isolated papules (lymphogenic spread). The centre of the lesion showed several flat elevations of a dark-red colour. *Examination*: *B. hansen* negative; leishmania parasites only demonstrated after repeated examinations; leishmania cultures positive; intracutaneous test with leishmania vaccine negative. *Biopsy*: tuberculoid granulation tissue.

The above was, therefore, a case of recurrent leishmaniasis with a positive leishmania-vaccine reaction, elicited on four occasions. Only after healing had begun, after prolonged treatment with Grenz rays, did the reaction gradually become positive, at first occupying an area of no more than 0.5 cm. in diameter, while after complete healing of the original lesion it was 3 cm. in diameter.

A similar lesion has been reported by Kardos (quoted from Rávnay, 1943) for actinomycosis, an originally negative intracutaneous actinomycin reaction, changing to positive after gold treatment. Fuhs (quoted from Rávnay, 1943) described a reversion of the trichophytin reaction in a favus patient after lichen favicus had appeared on the trunk and extremities. In a case of sarcoid a similar reaction was observed by Goldschmidt (1925).

THE MECHANISM OF THE REACTION

To discover the mechanism of the reaction the passive transfer test, according to Prausnitz-Kuestner, was set up in 11 cases with sera from two patients. One of them presented a scar of a leishmania lesion which he had had 30 years before, the other active recurrent leishmaniasis of seven years' duration. The Prausnitz-Kuestner test was carried out according to the prescriptions given by Urbach (1943).

In four cases the result was positive. This goes to prove that leishmania patients develop antibodies in their serum which, after having been transferred to a normal individual, may there give rise to a positive leishmania-vaccine reaction.

The allergic nature of the leishmania-vaccine reaction is thus demonstrated.

This is also borne out by many of the clinical features of the disease: (a) the reaction may appear as early as a few days after infection; (b) it remains positive throughout the duration of the disease, and for many years afterwards; (c) in recurrent leishmaniasis the reaction is stronger than in the classical type.

Without a detailed discussion on the relationship between immunity and the sensitivity towards a specific micro-organism, we should be inclined, in view of the positive results of the Prausnitz-Kuestner test and the clinical features outlined above, to regard the leishmania test as of an allergic nature.

As in the case of bacterial allergic reactions, there can be no doubt of certain relations of the test to immune processes, though the relationship is not absolute. Thus, it is especially in recurrent leishmaniasis that the highest sensitivity appears, although the immune reactions are no doubt impaired in such cases. The analogy to the luetin and tuberculin reaction is obvious.

SUMMARY

1. From a study of 434 cases of cutaneous leishmaniasis, the value is discussed of the various diagnostic methods, particularly the demonstration of parasites and the intracutaneous test.

2. That a skin-test would be useful is shown by the fact that the microscopical demonstration of parasites was positive in only 66.6 per cent. of cases of clinically verified leishmaniasis.

3. The specificity of the leishmania skin-test was 91.6 per cent. in a total of 261 cases tested, while its non-specificity was 6.3 per cent. in a control-series of 144 cases.

4. No difference was noted in the reaction of the skin to the vaccines prepared from different strains.

5. From the positive results of the passive Prausnitz-Kuestner transfer test and certain clinical facts, it appears that the reaction is of an allergic nature.

6. A peculiar case is reported, in which an originally negative reaction became positive after the lesion had healed.

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THE INFECTIVITY TO MAN OF A STRAIN OF *TRYPANOSOMA RHODESIENSE* TRANSMITTED CYCLICALLY BY *GLOSSINA MORSITANS* THROUGH SHEEP AND ANTELOPE: EVIDENCE THAT MAN REQUIRES A MINIMUM INFECTIVE DOSE OF METACYCLIC TRYPANOSOMES

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I. INTRODUCTION

The relationship of *Trypanosoma rhodesiense* to *T. brucei* has for long been a matter of controversy. German workers (Kleine, Taute, Hüber, Beck) maintained that the two were distinct species, *T. rhodesiense* being differentiated from *T. brucei* by its power to infect man (quoted from Wenyon, 1926). On the other hand, Bruce *et al.* (1914a), Kinghorn and Yorke (1913a), Wenyon (1926), Yorke, Adams and Murgatroyd (1930) and Duke (1935b) maintained that *T. rhodesiense* was the same as *T. brucei*, and was merely a strain of the latter which had become established in man. This hypothesis has been developed in detail by the latter workers.

As the result of their work on the *in vitro* action of human serum on one old and one recently isolated strain of *T. rhodesiense*, and on an old strain of *T. gambiense* (all the strains having been maintained by syringe passage through small laboratory animals), and on the known action of human serum on *T. brucei*, Yorke, Adams and Murgatroyd (1930) stated their views as follows:

'1. *T. rhodesiense* is synonymous with *T. brucei*, and the antelope and other game of Central Africa constitute, as was claimed by Kinghorn and Yorke, the reservoir.

'2. Normal human beings cannot be infected with the parasite derived from game—either by blood injection or by the intermediary of *Glossina*—because of the remarkable trypanocidal power of human blood (plasma).

'3. In individuals suffering from certain diseases, and probably also in those suffering from the effects of insufficient or improper dietary, the trypanocidal power of the blood is lost, and consequently such persons can be infected by *Glossina* which have drawn the virus from game.

'4. When once the game trypanosome has established itself in an individual of this kind, it by degrees becomes serum-resistant—a process which is accelerated if, for any reason, the cause of the initial decrease or absence of trypanocidal substance is removed and the serum gradually recovers its lost power.

'5. After it has sojourned in the human host for some time, the trypanosome is, like *T. gambiense*, definitely serum-fast, and consequently—unlike the parasite derived directly from game—infective for man.

'6. The serum-fastness is possibly preserved during the passage of the parasite through *Glossina*; and if so, the infection could spread from man to man through cyclically infected tsetse. If, however, this proves not to be the case, then the only obvious explanation of such localised epidemics of this form of the disease as have occurred, e.g., the Mwanza epidemic, is either that the fly transmits

mechanically the serum-fast parasite from man to man, or that some local condition exists, e.g., dietary deficiency, or hookworm disease, which so affects the population as to deprive many individuals of the protection due to the trypanocidal power of the blood.

7. If the parasite is passed on by tsetse to game or domestic animals instead of to man, it quickly loses its serum resistance—how quickly we do not yet know, but the available evidence suggests that the period is between one and two years—and so again becomes incapable of infecting normal man.

There is considerable epidemiological evidence that epidemics of Rhodesian sleeping sickness are caused by cyclically infected *Glossina*, and this view, as opposed to mechanical transmission, is now generally accepted. If a man, whose blood has no trypanocidal power (owing to disease, vitamin deficiency, etc.), is infected with *T. brucei* and his health and/or diet improves and the trypanolytic power of his serum returns, it is understandable that the trypanosome may develop serum-resistance. But where a population is suffering from disease and vitamin deficiency, and these conditions continue unabated after *T. brucei* has infected man (as they actually do in epidemics), the trypanolytic power of the blood will remain absent; and it is difficult to see how the trypanosome will develop a resistance to a factor which is not present, a serum-resistance which recently isolated strains of *T. rhodesiense* have been shown to possess in considerable degree (Fairbairn, 1933a).

The hypothesis makes two assumptions: first, that the resistance to human serum of *T. rhodesiense* is the basic factor governing its infectivity to man, which is very doubtful (Fairbairn, 1933b; Lester, 1933); and, secondly, that this resistance to human serum can be acquired and lost in a comparatively short space of time, which appears to be contrary to bacteriological principles.

Duke also maintained the hypothesis of the identity of *T. rhodesiense* and *T. brucei*. Three strains of *T. rhodesiense*, nos. 32, 38 and 26, had been isolated by one of us (H. F.) at the beginning of a major epidemic of Rhodesian sleeping sickness in Kibondo District. (There had already been 460 microscopically proved cases, and there were to be over 3,200 cases before the epidemic was brought under control.) Shortly after isolation these three strains were sent to Entebbe, where Duke investigated them as strains Tinde I, Tinde II and Tinde III (Duke, 1933b, 1935a, 1935b, 1937). In strains 32, 38 and 26 it was shown (Fairbairn, 1933a) that the trypanosomes were resistant to human serum *in vitro* on isolation, and that this resistance was still present after 4, 12 and 21 syringe passages through rats over periods of 115, 182 and 370 days. Strain 26 (i.e., Tinde III) was still infective to man after 19 rat passages by syringe over 345 days, when the resistance to human serum *in vitro* appeared to be low (Fairbairn, 1933b).

As a result of his experiments, Duke maintained the view of the close relationship of *T. rhodesiense* and *T. brucei* (Duke, 1935b). In the discussion of his results (Duke, 1935a) he noted the loss of the power to infect man of two strains of *T. rhodesiense* while actually under observation.

From an examination of the tables, however, only one strain, Tinde I, became non-infective, eight men bitten failing to be infected. The other strain, Tinde III, passaged through bushbuck I remained infective to man; and it was only the line maintained by syringe passage in 14 guinea-pigs over 18 months which became non-infective. As Duke himself stated that maintenance of trypanosomes in guinea-pigs was liable to impair the cyclical transmissibility by tsetse—and possibly to impair the ability to infect man (Duke, 1935a)—it cannot in fairness be said that this strain as a whole had lost its infectivity to man, merely because the line through guinea-pigs became non-infective.

He went on to say: 'Kleine and his co-workers believe that *T. gambiense* and

T. rhodesiense are one and the same species, zoologically distinct from *T. brucei* and characterised by its power to infect man. But we have here a strong indication not only that there is a great difference between the two trypanosomes of man in this important respect, but also definite proof that pathogenicity to man is not entitled to specific significance.' Further on he remarked: 'we have now seen that the distinction between the two latter [i.e., between *T. rhodesiense* and *T. brucei*] is purely arbitrary, and rests on the exaggerated importance hitherto attached to pathogenicity to man. Now that this character is known to be neither absolute nor stable, there is no longer justification for the common assumption that every strain of *T. rhodesiense* found in man has necessarily had previous knowledge of man.' Two years later, however, Duke (1937) found that strain Tinde I was again infective to man. Neither strain Tinde I nor Tinde III had therefore lost its pathogenicity, and yet he did not make any alteration in his hypothesis.

It will be seen that the hypothesis that *T. rhodesiense* and *T. brucei* are transmutable rests upon two assumptions: (a) that if *T. rhodesiense* were maintained for a short period (1-2 years) in game it would lose its resistance to human serum and hence its infectivity to man, and would revert to *T. brucei*; and (b) that the infectivity to man of *T. rhodesiense* is not a stable characteristic, is easily lost, and is not of valid specific significance.

II. THE INFECTIVITY TO MAN OF A STRAIN OF *T. RHODESIENSE*

On October 21st, 1934, a strain of *T. rhodesiense* was isolated by feeding laboratory-bred *Glossina morsitans* on a new untreated case of Rhodesian sleeping sickness (Corson,

TABLE
The infectivity to man of the strain of *T. rhodesiense*

Volunteers bitten by cyclically infected flies from	1936			1937			1938			1940		
	No. of men			No. of men			No. of men			No. of men		
	Bitten	In- fected	% infected	Bitten	In- fected	% infected	Bitten	In- fected	% infected	Bitten	In- fected	% infected
Main Lines												
Sheep	2	2	100	2	2	100	9	1	11.1
Sheep 254	5	2	...
Thomson's gazelle...	2	2	...
Reedbuck	2	1	50	1*	0	64.7	8	6	75
Impala	2	1		—	—		2	1	
Bushbuck	2	1		—	—	
Dik-dik	12	9
Steinbuck	4	2
Subsidiary Lines												
(A) <i>From Antelope</i>												
Eland 5	2	0	—
Sheep ex eland	12	6
Impala 7
Man ex T.G. 75
(B) <i>From Sheep</i>												
Monkey
Sheep M2 B ex man
T.G. ex sheep
Reedbuck 12 ex sheep
Bush-pig 4 ex R.B. 12
R.B. ex sheep

* This test was actually done on 15.1.39.

1936a), and the strain has since been passaged continuously through sheep by cyclically infected *G. morsitans*. On November 17th, 1936, a reedbuck was infected by a fly from sheep 165, the 12th sheep host, and a second line of the strain has since been passaged in the same way through a variety of antelope.

Tests of the infectivity to man of these two lines have been made at intervals, and the results up to January, 1939, were reported in a series of papers (Corson, 1936a, 1937a, 1937b, 1938a, 1938b, 1939a, 1939b; Fairbairn, 1937). The object of the present communication is to report the tests of the infectivity to man of the strain in the years 1940-45. In order to give a complete picture, the transmission of the strain from the beginning of the experiment is shown in Appendix D, with the details of all the human volunteer tests which have been done up to date.

The hungry fly was placed upon the outer surface of the man's upper arm, and was allowed to remain there until it had had its fill of blood, i.e., a 'feed' as opposed to a bite (a probe). Six men who were merely probed by the fly and not infected are not included in the results; but four men (volunteers 53, 72, 79 and 314) probed and infected are included. In every case where a man was apparently not infected, his blood was inoculated into white rats, so as to exclude the possibility of missing a 'cryptic' infection.

The results of the tests are summarized in Table I. From 1941 onwards comparatively large numbers of men were used for each test so that the results might be analysed statistically, and this analysis is shown graphically in fig. 1.

It will be seen that, 10½ years after the strain of *T. rhodesiense* had been removed

I
transmitted cyclically by *G. morsitans* through various hosts

1941			1942			1943			1944			1945		
No. of men			No. of men			No. of men			No. of men			No. of men		
Bitten	In-fected	% infected	Bitten	In-fected	% infected	Bitten	In-fected	% infected	Bitten	In-fected	% infected	Bitten	In-fected	% infected
11	4	36.3	10	7	70	11	8	72.7	13	8	61.5	14	13	92.9
10	9	90	10	5	50	11	3	27.2	14	3	21.4	10	6	60
...
...
...
...
...
...
...
...
10	7	70
...	13	4	30.8
20	11	55	10	6	60	12	7	58.3	14	4	28.5	13	5	38.4
9	5	54.4
...	10	3	30	6	5	83.3
...	10	7	70
...	10	6	60
...	10	2	20	8	3	37.5

Only the results of the first bites are shown.

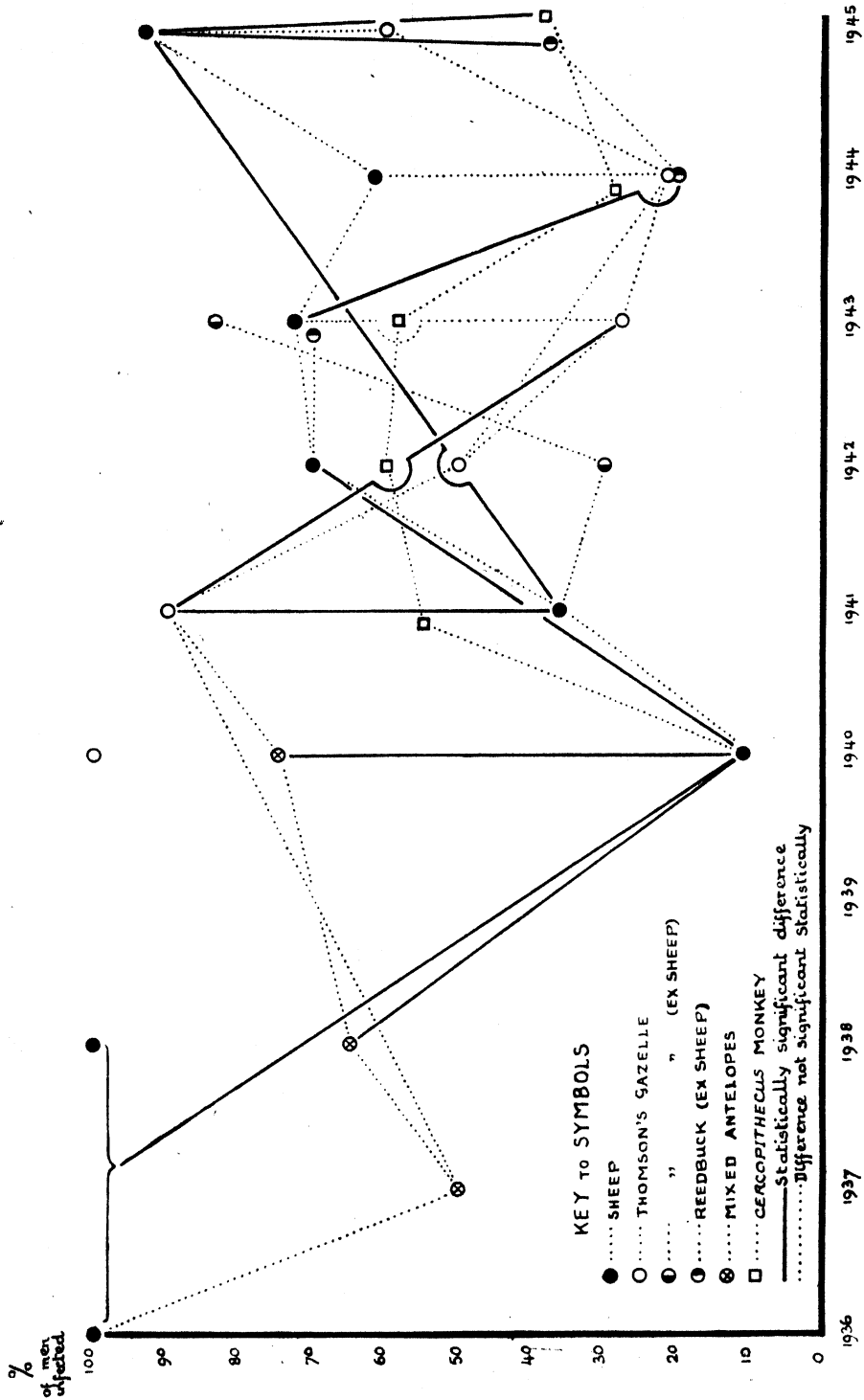


FIG. 1. Diagram illustrating the results of the statistical analysis of the infectivity to man of the various lines.

from man and had been passaged by cyclically infected *G. morsitans* through a variety of animals, it was still highly infective to man.

The infectivity of the various lines, however, has not been constant. Statistical analysis showed that in the sheep line the infectivity fell markedly and significantly by 1940, but rose again gradually, until by 1945 it was as infective to man as in the years 1936-38, and was significantly higher than in 1940. During 1940 and 1941 the infectivity of the sheep line was significantly lower than that of the antelope line.

The antelope line, *taken as a whole*, showed no significant alteration in infectivity over the period 1937-40; but, as by 1940 maintenance in the single species, sheep, appeared to have depressed infectivity, it was decided to abandon the maintenance of the antelope line in a mixture of hosts and to study rather the influence exerted by a single species. The antelope line from 1940 onwards was therefore passaged solely through Thomson's gazelle; and it was then found that infectivity dropped significantly between 1941 and 1944. There was a non-significant rise between 1944 and 1945, but it was enough to make the infectivity in 1945 no longer significantly different from that of 1941.

When the 1940 tests from sheep 275 and 277 showed that the infectivity to man was low, sheep 254, infected on April 14th, 1939, was still alive. Fly EC.5 was infected from this latter sheep in May, 1940 (i.e., 13 months later), and it infected two men out of five whom it bit; but the numbers involved are too small for statistical comparison.

As maintenance in different species appeared to influence infectivity markedly, it was decided to start a series of lines in other hosts.

1. From the one man infected in the 1940 tests on the main sheep line, a line through sheep (the M/sheep line) was started by feeding a box of clean laboratory-bred *G. morsitans* on the patient. This was to see if the single passage through man, and the contact of the trypanosomes with human serum, would raise the infectivity of the strain to man.

2. In 1940, when the infectivity of the sheep line was very low, a line through *Cercopithecus* monkeys was started from sheep 275, as the work of Duke (1935*b*) appeared to show that *Cercopithecus* monkeys had a marked influence in raising the infectivity of a trypanosome to man.

3. In 1941, while the infectivity of the sheep line was still comparatively low and that of the antelope line was high, a line in Thomson's gazelle (T.G. ex sheep) was started from sheep 289, to study its influence on infectivity.

4. The first failure to infect man occurred in 1937 with fly BP. 35 from impala 2, and further failures occurred with infected flies from eland 5 and with flies from sheep 227 and 228, themselves infected from eland 5. The trypanosome infection in impala and eland appeared to run a similar chronic course, the animals eventually throwing off the infection; and these two species appeared to have depressed infectivity. Eland were not available, but from 1940 a line was maintained in impala (from impala 4), to see if the above results would be repeated.

Other lines were taken off the sheep line and passaged through reedbuck and bush-pig for short periods, to study the influence of these hosts.

The results are shown in Table I and fig. 1.

By 1941 the infectivity of the main sheep line had risen somewhat; and, although the infectivity to man had increased in both the monkey line and the M/sheep line, this increase was not significant.

In 1937, when the antelope line was originally started, there appeared to be a depression

of infectivity compared with the main sheep line, although the result was not statistically significant on the numbers available. The infectivity in 1942 of the T.G. ex sheep line was low, but not significantly lower than in the sheep line in 1941 from which it had been derived. However, there was a significant decrease in infectivity on comparing reedbuck in 1944 with the main sheep line in 1943 from which it was taken.

Passage of the trypanosome through four impala hosts over a year did not appear to depress infectivity, as 70 per cent. of the men tested were infected.

In the monkey line, the infectivity increased immediately and remained at 50–60 per cent. for three years. By the fourth year, 1944, it had dropped to 30 per cent.—a decrease which was not significant on the numbers available—and it had risen to 38.4 per cent. by 1945. In 1945, however, the monkey line was significantly less infective than the sheep line.

The question of why change of host in some cases appears to depress and in other cases to enhance infectivity requires further investigation, as also does the matter of the alteration in infectivity which takes place during maintenance in single species.

The resistance of the strain to human serum *in vitro* was tested in 1936 at the 6th, 7th, 9th and 10th sheep passages (Fairbairn, 1937), and was again tested in 1938 by Hawking (1940) at the 21st passage from impala 3. There was no apparent diminution in the resistance of the strain to human serum *in vitro*.

The results recorded above show that the strain of *T. rhodesiense*, taken as a whole, was still highly infective to man 10½ years after isolation, and that it showed no signs of reverting to *T. brucei*, as Yorke *et al.* postulated would happen. Although by 1940 the infectivity of the sheep line was low, it was also being transmitted with difficulty (Table II); and it could not be said to be reverting to a *brucei* type, but merely that it was becoming non-infective and non-transmissible. By 1945 the infectivity to man of the sheep line had markedly increased, and it was again easily and, for this line, highly transmissible.

Secondly, though the infectivity to man of the various lines varied within wide limits, it was never lost—a result contrary to the hypothesis of Yorke *et al.* and of Duke. Infectivity to man of the strain in all lines has been present, and could be demonstrated, provided that a sufficient number of men were tested; and there is another explanation for a failure of some men to be infected, other than that *T. rhodesiense* was reverting to a non-infective *T. brucei*.

III. THE FAILURE OF MAN TO BE INFECTED

In 1940 volunteers 61 and 63, who were not infected by fly EC. 5 from sheep 254, were rebitten by fly EA. 4 from Thomson's gazelle. Both men fell ill, with a characteristic reaction at the site of the second bite. This fly had thus infected all four men which it had bitten. (This result is not shown in Table I, which gives the results of first bites only.)

A re-examination of Corson's records showed that he had had a similar experience with volunteers 8, 9, 23 and 31 (Corson, 1938*a*, 1938*b*, 1939*a*). Volunteer 23 fell ill on a rebite by a different fly from the same host; volunteers 9 and 31 fell ill on rebites by flies from other hosts; while volunteer 8 was infected on a second rebite (i.e., three in all), again by a fly from another host.

With reference to volunteer 23 (Masere Masesa), Corson (1938*b*) wrote: 'The case of Masere Masesa seems to show that a man may resist infection from the bite of one fly and become infected from the bite of another fly which had got its infection by feeding

on the same animal and at the same time as the former fly. . . . It appears as if fly CG 25 carried more infective trypanosomes than fly CF 11 or at least injected more infective trypanosomes into the volunteer. Nothing is known of changes in a person's resistance to infection with *T. rhodesiense* but it is hard to think that this volunteer was less resistant on March 24 than on March 13.' From a reference in a previous paper (Corson, 1937c) it would seem that when Corson used the phrase 'more infective trypanosomes' he was referring to trypanosomes of greater infectivity, i.e., he was referring to a difference in quality of the trypanosomes, and did not mean a mere increase in dose of infective trypanosomes. Corson (1937c) had also discussed the possibility that a strain of *T. rhodesiense* might be of heterogeneous composition in regard to infectivity to flies and vertebrates, and that, when two flies became infective after feeding on the same animal, one of them might have become infected with trypanosomes of greater infectivity than the other. Finally, in summing up the results of his work at this laboratory, Corson (1939c) wrote: 'It was found that men differed in resistance, some not becoming infected, and it appeared also that individual flies differed in respect of the infectivity to man of the trypanosomes they carried.'

In order to test whether individual flies did differ in respect to the infectivity to man of the trypanosomes they carried, the 1941 and subsequent tests were planned as follows. Any man who was not infected by the bite of an infected fly was rebitten after an interval by the same fly, or by another fly from the same isolation, or by a fly from a different isolation from the same host. Should any man fail to be infected on such rebite, he was to be rebitten by a fly carrying what was then thought to be a more highly infective trypanosome (e.g., a man who failed to be infected by a fly from sheep was rebitten by a fly infected from monkey or Thomson's gazelle).

The results were as follows.

Rebites by the Same Fly. Of 35 men who were rebitten by the same fly which had failed to infect them originally, 12 were infected. They were distributed thus:

Sheep line	1941	Volunteers	108a, 113a, 115a
			1942	"	128a
Monkey line	1941	"	88a, 93a
			1943	"	176a
Thomson's gazelle line			1942	"	158a, 162a
T.G. ex sheep line	1942	"	139a, 141a, 144a

It will be seen that for each host these successful rebites occurred at a time when the infectivity of the line concerned was between 20 per cent. and 60 per cent.

Rebites by Another Fly from the Same Isolation. Of 13 men who were rebitten by another fly from the same isolation, four were infected:

Sheep line	1941	Volunteers	114a
Monkey line	1941	"	99a, 100a
			1942	"	152a

Rebites by a Fly from a Different Isolation from the Same Host. Of 14 men who were rebitten by a fly from a different isolation from the same host, six were infected. They were:

Sheep line	1938	Volunteers	23a
			1941	"	112a
			1943	"	190a
Impala line	1941	"	81b
T.G. ex sheep line	1942	"	145a, 146a

Rebites by a Fly Infected from Another Host. Of 18 men who were rebitten by a fly infected from a different host, seven were infected. They were :

- Volunteer 8b infected 1937 from reedbuck (negative to eland twice).
 " 9a " 1937 " " (negative to eland).
 " 31a " 1938 " dik-dik (negative to sheep ex eland).
 " 61a and 63a infected 1940 from Thomson's gazelle (negative to main sheep line).
 " 121a infected 1941 from main sheep line (negative to M/sheep line).
 " 194b " 1943 " Thomson's gazelle (negative to reedbuck twice).

It is conceivable that two flies fed at the same time on the same host might be infected with trypanosomes of different infectivity to man (i.e., a difference in 'quality') if the strain were heterogeneous; and this hypothesis is even more tenable when the two flies compared are from different isolations from the same host. But, when 12 out of 35 men

TABLE II
The transmissibility and infectivity of the sheep, Thomson's gazelle and monkey lines

	1934	1935	1936	1937	1938	1939	1940	1941	1942	1943	1944	1945 (to May)
<i>Sheep Line</i>												
No. of transmission-experiments ...	1*	3	13	7	6	8	6	14	8	4	11	7
No. of flies surviving to be examined ...	172	156	1,000	430	594	483	415	659	266	112	290	143
No. of flies with positive salivary glands...	4	9	56	11	13	5	14	27	11	4	12	4
Percentage of flies positive ...	2.3	5.7	5.6	2.6	2.2	1.0	3.4	4.1	4.1	3.6	4.1	2.8
Percentage of men infected on first bite...	—		100.0		100.0	—	11.1	36.3	70.0	72.7	61.5	92.9
<i>Thomson's Gazelle Line</i>												
No. of transmission-experiments ...						3	5	10	14	4	16	6
No. of flies surviving to be examined ...						210	375	551	601	149	482	171
No. of flies with positive salivary glands...						15	19	26	27	2	14	7
Percentage of flies positive ...						7.1	5.0	4.7	4.5	1.3	2.9	4.1
Percentage of men infected on first bite ...							100.0	90.0	50.0	27.2	21.4	60.0
<i>Monkey Line</i>												
No. of transmission-experiments ...							1	2	7	3	11	11
No. of flies surviving to be examined ...							56	86	211	158	351	282
No. of flies with positive salivary glands...							4	4	10	3	17	16
Percentage of flies positive ...							7.1	4.6	4.7	1.9	4.8	5.7
Percentage of men infected on first bite ...								55.0	60.0	58.3	28.5	38.4

* The original isolation from man on 21.10.34.

N.B. From 1934-39 the percentage of flies with infected salivary glands was calculated from dissections and isolations; from 1940 onwards the percentage was calculated from flies found to be infected by isolation on rats only.

were infected when rebitten by the same fly after an interval of 13-30 days, it did not appear that a differential quality of the infectivity of the trypanosome was an adequate explanation of the facts. For this would presume that the infective quality of the metacyclic trypanosome could alter while it was resident in the tsetse; or that man's resistance, trypanolytic power of serum, etc., could diminish within 2-4 weeks.

On the other hand, it was possible that the trypanosomes were homogeneous, and that infection of man was merely a matter of adequate dosage of metacyclic trypanosomes, this dosage being dependent upon the ability of the trypanosomes to develop in adequate numbers in the salivary glands of the fly.

Table II shows the transmissibility for each year of the sheep, Thomson's gazelle and monkey lines, together with the percentage of volunteers infected on the first bite. (Experiments which failed to yield a salivary-gland-infected fly have been excluded.) The infectivity of the sheep line in 1940 was tested in February and March, the two flies used having fed on their hosts in January and February respectively; and, when it was found that the infectivity was low, it was noted that the transmissibility of the line during 1939 was also very low.

The results in Table II recorded up to 1945 have been examined statistically. As the infectivity tests have been done each year in the period February-May as a rule, and as the great majority of the transmission-data were obtained later in the year, the transmissibility of one year was tested against the infectivity of the next year. For the three lines, sheep, Thomson's gazelle and monkey, the values of the correlation coefficient were +.8625, +.5507 and +.7313 respectively, which are not significant on the five pairs of observations available in each line. Taken together, the results by the 'z' test give a correlation between transmissibility and infectivity of the strain ($P = .02$). In 1940 this evidence was not available, but, as mentioned above, the low transmissibility of the sheep line in 1939 led us to suspect that the salivary glands of the flies might have been lightly infected, that the flies inoculated comparatively small doses of metacyclic trypanosomes, and that, if man required a minimum infective dose, this might not always have been inoculated, and hence the low infectivity.

Bound up with dosage is the question of man's resistance to infection. Up to 1945 336 volunteers had been bitten, of whom only 52.7 per cent. were infected on the first bite. A number of men who failed to be infected were rebitten once or more, giving a total of 416 tests and a total infection-rate of 61.6 per cent. of the original 336 volunteers. The results are shown in Table III.

TABLE III

The number of volunteers bitten and rebitten, by the same or different flies, and the proportion of the men infected

	No. of men bitten	No. infected	No. not infected	Percentage infected
Volunteers bitten	336	177	159	52.7
Negative on first bite, rebitten	66	26	40	39.3
" second " "	13	3	10	23.0
" third " "	1	0	1	—
Total	416	206	210	49.5

Of the original 336 men, 61.6 per cent. were infected after rebites.

It will be seen that on each rebite a diminishing proportion of men became infected. In view of the fact, discussed below, that the older the fly the better is its chance of inoculating a high dose of metacyclic trypanosomes, this table proves that some men have a high resistance, either natural or produced by 'vaccination,' as suggested by Duke (1935a, 1935b). The following case-histories illustrate this point:

Volunteer 69 was bitten by fly EO. 10 on 17.2.41; he was rebitten by fly ES. 12 on 3.3.41, and was rebitten by fly ER. 36 on 31.3.41, and was not infected. On 8.4.41 he was inoculated with 102,000 blood trypanosomes from a rat and was not infected. On 29.4.43 he was inoculated with 1,067 metacyclic trypanosomes and was not infected.

Volunteer 83 was bitten by fly EQ. 69 on 17.2.41; he was rebitten by the same fly on 5.3.41, and was rebitten by fly EU. 12 on 22.3.41, and was not infected. On 8.4.41 he was inoculated with 102,000 blood trypanosomes from a rat and was not infected.

Volunteer 98 was bitten by fly ER. 41 on 15.2.41; he was rebitten daily by the same fly on 1-5.3.41, and was rebitten by fly ER. 36 on 19.3.41, and was not infected. On 8.4.41 he was inoculated with 102,000 blood trypanosomes from a rat and was not infected. On 26.4.43 he was bitten by fly IO. 10 and again was not infected.

Cercopithecus monkeys infected with *T. rhodesiense* have an incubation-period of 5-7 days, trypanosomes are constantly present in the blood, and, as Corson (1938d) showed, the monkeys invariably die, with involvement of the central nervous system.

In monkeys 266, 270 and 336, however, we have had examples of 'resistance' or 'immunity' to infection with *T. rhodesiense*. The position of these monkeys in the transmission line, and their relation to each other, is shown in fig. 2.

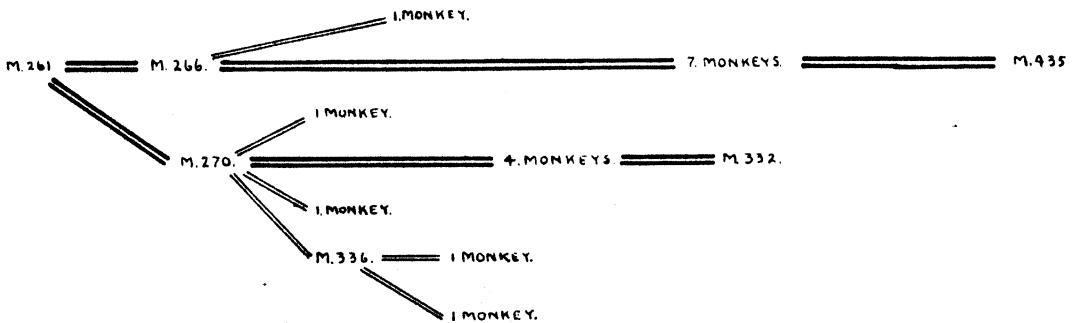


FIG. 2. Diagram illustrating the position of monkeys 266, 270 and 336 in the main monkey transmission line. Flies infected from monkeys 261, 332 and 435 were used to test the infectivity to man of the line in 1941, 1942 and 1943 respectively.

Monkeys 266 and 270 were infected from monkey 261 used in the 1941 tests of infectivity of the line to man, and monkey 336 was infected from monkey 270. Their histories are briefly as follows:

1. *Monkey 266.* Boxes of flies from monkey 261 were applied on 6.12.40, and its blood was first positive on 27.12.40. From that date until 9.11.42, when its blood was last microscopically positive, trypanosomes were only found occasionally, and of two rats inoculated on 9.11.42 only one was infected. Between 18.11.42 and 6.5.43 no trypanosomes were ever seen, and of 15 pairs of rats inoculated at intervals with blood none was infected.

On 5.6.43 the monkey was inoculated with $\frac{1}{2}$ c.cm. of heavily infected blood from rat 4767 (T.G. line), and, as it was still negative on 23.6.43, it was bitten by fly JJ. 18 ex T.G. 56. Trypanosomes were seen in its blood on 15, 16 and 17.8.43, but from that date its blood has been microscopically negative despite repeated examinations, and none of the 32 pairs of rats inoculated from it has been infected. The monkey shows no signs of being ill, and it is now $4\frac{1}{2}$ years since it was first infected.

2. *Monkey 270*. Boxes of flies from monkey 261 were applied on 22.1.41, and its blood was first positive on 4.2.41. Trypanosomes were only seen occasionally in its blood between then and 25.5.42, which was the last date on which its blood was microscopically positive. Between 6.5.41 and 21.7.41 five attempts at transmission, using 561 flies, failed. On 1.7.42 two rats were inoculated and one was infected, but of 47 pairs of rats inoculated at intervals since then none has been infected. The monkey apparently is cured.

3. *Monkey 336*. Boxes of flies from monkey 270 were applied on 12.1.42, and its blood was positive on 30.1.42. Trypanosomes were present during May and June, 1942, up to 2.7.42, and then were only found on 26.11.42 and 5.2.43, despite weekly examinations. From this last date 30 pairs of rats have been inoculated with its blood, but none has been infected.

Transmissions were ultimately obtained from these three monkeys, and the subsequent history of the line showed nothing unusual, the incubation-period, character of the disease and length of life of the monkeys being the same as previously.

Three *Cercopithecus* monkeys have recovered naturally from infection with *T. rhodesiense*, and one of them has recovered from a reinfection. These monkeys must have had a naturally high resistance or immunity; and it has been seen that nearly 40 per cent. of men tested have failed to be infected. If immunity is present normally in a proportion of men and animals, it should be possible to develop it artificially in all individuals, and this subject requires urgent investigation by a competent immunologist.

It may be argued that the failure of some men to be infected was due to the fact that an infected fly on occasions did not extrude metacyclic trypanosomes when it fed. Corson (1932), using a single infected fly, found that successive bites (i.e., probes) caused infection in laboratory animals in every case, but he only did 12 tests. This, however, has not been our experience. Of 247 rats merely probed into or bitten by proved infective flies, 22 were not infected (6.3 per cent.); while of 861 rats *fed upon* by infective flies seven were not infected (0.8 per cent.). It is therefore clear that an infected fly does not necessarily extrude metacyclic trypanosomes when it bites, or even when it feeds; but the percentage of failures in rats is too low to explain the fact that 47.3 per cent. of men failed to be infected when fed upon the first time by a known infective fly. There has been no evidence that a salivary gland infection with this strain of *T. rhodesiense* has died out during the fly's life.

If, however, it were proved that man required a minimum infective dose of metacyclic trypanosomes in order to be infected, that the M.I.D. was of a high order, that some men required a larger dose of metacyclic trypanosomes to be infected than others, and that the inoculation of this dose depended upon the transmissibility of the strain, then a concise and logical explanation of the facts would be available.

IV. THE MINIMUM INFECTIVE DOSE OF METACYCLIC TRYPANOSOMES REQUIRED TO INFECT MAN

A review of the literature available produced very little information as to the number of trypanosomes inoculated when a fly fed. Rodhain, Pons, Vandenbranden and Bequaert (1912) induced *G. morsitans* infected with *T. brucei* to feed through a membrane on citrated blood, and they estimated that a single fly was able to inject more than 1,562 metacyclic trypanosomes while feeding. Van Hoof, Henrard and Peel (1937a) using the same technique and a single *Glossina* (species unstated) infected with *T. gambiense* in three experiments found 12, 0 and 2 trypanosomes injected. Lloyd and Paisley (1929) also induced infective flies carrying *T. brucei* to feed through a membrane from a small quantity of serum. The fluid remaining was examined for the injected trypanosomes, and, although

TABLE IV
The number of metacyclic trypanosomes counted on a series of slides probed consecutively

Year	Infesting host	Fly	Slides													Total of metacyclic trypanosomes extruded
			1	2	3	4	5	6	7	8	9	10	11	12	13	
1942	Sheep 297	GA. 10	170	N.S.	165	90	9	19	N.S.	—	—	—	—	—	—	453
1943	"	GA. 33	84	178	2	38	4	—	—	—	—	—	—	—	—	306
	Sheep 327	*IK. 26	1,453	157	15	32	79	—	—	—	—	—	—	—	—	1,736
"	"	*IN. 11	1,036	3	241	54	35	86	180	2	N.S.	231	7	78	—	1,953
1942	T.G. 24	GD. 10	380	495	96	257	209	116	93	183	36	97	50	N.S.	—	2,012
"	T.G. 27	GE. 14	N.S.	982	1,738	117	458	671	N.S.	1,129	21	103	—	—	—	5,219
"	"	"	N.S.	N.S.	1,346	5	—	—	—	—	—	—	—	—	—	1,351
"	"	GE. 33	306	383	307	975	252	N.S.	1,438	326	10	1,535	—	—	—	5,532
"	"	GF. 2	234	127	57	27	4	78	19	4	20	18	—	—	—	588
"	"	"	253	5	2	16	41	8	66	47	13	4	82	—	—	537
"	"	"	1,794	700	26	185	178	46	58	123	525	99	—	—	—	3,734
"	"	GF. 30	3,809	627	778	36	29	79	84	51	36	64	—	—	—	5,693
"	"	"	211	2,385	2,734	N.S.	170	55	719	3	6	2	—	—	—	6,285
"	"	GF. 36	1,431	1,483	1,365	157	34	—	—	—	—	—	—	—	—	4,470
"	T.G. 28	GG. 32	1,119	57	12	90	8	24	19	5	47	27	—	—	—	1,408
"	"	GJ. 1	12	3	94	67	60	16	—	—	—	—	—	—	—	252
"	"	"	N.S.	0	109	16	201	—	—	—	—	—	—	—	—	326
"	"	GJ. 19	129	71	N.S.	11	N.S.	N.S.	14	—	—	—	—	—	—	225
1943	T.G. 39	*IO. 21	11	N.S.	N.S.	0	0	0	2	0	3	2	—	—	—	18
"	"	"	4	26	10	9	1	12	2	3	1	N.S.	—	—	—	68
"	"	"	3	3	0	2	6	0	8	6	0	4	—	—	—	32
"	"	"	5	0	0	1	0	0	1	0	0	0	0	2	0	9
"	"	"	0	0	1	0	1	0	2	3	5	5	N.S.	1	—	18
"	"	"	N.S.	2	2	0	N.S.	1	1	0	0	0	—	—	—	6
"	"	*IO. 10	N.S.	40	125	—	—	—	—	—	—	—	—	—	—	165
"	"	"	296	470	533	—	—	—	—	—	—	—	—	—	—	1,299
"	"	"	908	8,575	1,860	—	—	—	—	—	—	—	—	—	—	11,343
"	T.G. 51	KB. 42	8,155	2,075	1,344	—	—	—	—	—	—	—	—	—	—	11,574
"	"	"	1,692	296	167	867	—	—	—	—	—	—	—	—	—	3,022
"	"	"	956	56	79	N.S.	257	23	63	—	—	—	—	—	—	1,434
"	"	"	1,168	33	1,906	N.S.	140	—	—	—	—	—	—	—	—	3,247
1941	Monkey 266	FN. 22	113	53	92	166	57	11	—	—	—	—	—	—	—	492
1943	Monkey 441	*IJ. 49	1,561	1,298	629	227	106	N.S.	296	284	342	175	289	348	239	5,794

* Fly used in tests of infectivity of the strain.

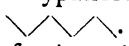
N.S. = No salivation on the slide.

these were found, they were present in such small numbers that no estimate could be made.

In August, 1941, Burt (1946*b*), modifying a method used by Bruce *et al.* (1914*b*), was able to count the number of metacyclic trypanosomes extruded by an infected tsetse-fly while attempting to feed. A series of clean microscope slides were smeared with a thin film of fresh egg-albumen, which was allowed to dry. The fly was contained in a wide-mouthed bottle, the mouth of which was covered with mosquito-gauze. The bottle being held mouth downwards in a rack, a guinea-pig was brought near, and, as the hungry fly extruded its proboscis through the gauze, one of the albumen-covered slides was interposed between it and the guinea-pig, and the fly probed on to the surface of the slide. It was found that, once the fly had started to probe on to the slide, the bait-animal could be removed and the fly would continue to probe the slide just as well as if the guinea-pig had been near. Such a 'probe' usually lasted 30 seconds before the fly withdrew its proboscis. When it again attempted to feed, a second slide was interposed, and again the fly probed on to it. In this way up to 13 slides have been probed upon consecutively, before the fly was given a full feed on a rat, which always became infected. An illustrative series of such probes by infected flies is shown in Table IV.

There is now a large amount of data available on this point—too extensive for it all to be published here; but the results recorded above are a representative sample. Occasionally a fly appeared to probe quite satisfactorily on the slide, and yet, when the slide was stained and examined, either no trace of salivation was found or only dry scratches (and hence no trypanosomes). Such an occurrence has been recorded as 'no salivation' (N.S.). This failure to salivate when a fly probed on to a slide may afford the explanation of why some rats failed to be infected when merely probed into by an infected fly. On the other hand, it was found occasionally that a probe of only a few seconds was sufficient for over 1,000 trypanosomes to be extruded.

This table brings out a number of important points:

1. Flies from the same isolation, i.e., fed at the same time on the same host, did not have their salivary glands infected to the same extent (compare flies GE. 14 and GE. 33, GF. 2 and GF. 30, and IO. 21 and IO. 10).
2. In a probe-test by the same fly on different days the number of trypanosomes extruded varies, sometimes enormously (see flies GE. 14, GF. 2 and IO. 10).
3. During a single test on a series of slides, the number of trypanosomes extruded was not constant, but fluctuated in a series of waves, such as . It seems as if there were a certain number of trypanosomes readily available for inoculation with the saliva, and that when this saliva with trypanosomes was exhausted there was a muscular effort of the salivary glands bringing up more saliva and trypanosomes, the number of the latter extruded gradually tailing off over a series of slides, until there was another effort bringing up more saliva and trypanosomes. The bearing of this point on the physiology of salivation of the fly, and its power to infect man, is discussed below.
4. Isolations at different periods from sheep and Thomson's gazelle yielded flies whose salivary glands had markedly different numbers of trypanosomes available for inoculation. Of flies isolated from the various Thomson's gazelles, fly GJ. 1 (used in the 1942 tests) extruded fewer trypanosomes than the flies in the GD, GE and GF isolations of November-December, 1941; while fly IO. 21 (used in the 1943 tests) had even fewer trypanosomes available than fly GJ. 1.

The probe technique is simple to use for the immediate isolation of infected flies. It has the advantage that isolation can be effected without waiting for a rat to show up positive. Flies isolated by this means were confirmed on one or more rats before being used for volunteer tests.

The question naturally arose as to what relation these trypanosome counts on slides had to the number of trypanosomes inoculated when a fly fed on man. The answer to this question depended upon the answer to a further question: 'What was the action of the salivary glands during the act of feeding by the tsetse? Was the secretion of saliva continuous or intermittent during feeding, or did it take place only once, just after the probing of the skin and prior to the sucking up of the blood?' For, if the injection of saliva was continuous or intermittent during feeding, then a moderately infected fly had a greater chance of inoculating an infective dose of trypanosomes than if the injection of saliva (and trypanosomes) only occurred once at the beginning of the act of feeding. In actual practice, the dose of metacyclic trypanosomes inoculated may also vary with the feeding habits of the flies, for some flies probe once and feed full in less than a minute, while others probe repeatedly and then take several minutes to have a full meal of blood.

Van Hoof, Henrard and Peel (1937a), as a result of their work on the number of trypanosomes inoculated on a bite, believed that the blood ascending the proboscis when the fly began to feed stopped the expulsion of infective saliva, and that if a large number of trypanosomes were discharged it was only at the time when the proboscis pierced the integument. Gordon and Lumsden (1939), observed the acts of feeding of *Aedes aegypti*. They state that 'mosquitoes which were observed to feed for a period up to 30 seconds seldom proved on dissection to have taken up blood, although the reaction produced by the bite was evidence of the injection of salivary secretion. Such observations suggest that part at any rate of the salivary secretion is injected into the tissues before the blood-supply is reached. . . . It is likely that salivary secretion is injected at all stages of penetration by the fascicle . . . we are inclined to think that salivary secretion is injected during the whole act of biting and is deposited in the tissues, in the lumen of the capillaries, and in any extravasation of blood produced by the mosquito.' In a personal communication, Professor Gordon said that he was unable to state whether or not the flow of saliva ceased during feeding. Lester and Lloyd (1928) said: '. . . it is clear that the injection of the salivary secretion into the host is not a necessary preliminary to the act of feeding, but rather in the nature of an accident, because the salivary secretion and blood mix at the very tip of the proboscis. We have attempted to show how this occurs in a diagram in which a cloud of salivary secretion is depicted as escaping out of the hypopharynx into the tip of the proboscis, which rests in the blood stream. Most of it passes back up the proboscis mixed with the indrawn blood, but some is carried away by the blood brushing past it and so is lost to the fly. It is this escape which causes the wheal at the site of the bite.'

The latter authors would, therefore, appear to consider that salivation occurred during the whole period of feeding. The following experiment was carried out to investigate this point. The mouth of a bottle containing an infected fly was covered with a fresh rat-skin. When the fly's proboscis pierced the skin it was made to probe on to a series of albumen-covered slides, following one another in rapid succession. In this way a series of eight slides were probed upon in 2 minutes 40 seconds during a single insertion of the proboscis through the skin, a time comparable to that of an average feed

upon man. Subsequent examination showed copious saliva and a varying number of trypanosomes on each slide. The experiment was repeated several times, always with the same result.

We therefore proceeded on the assumption that salivation *was* continuous during feeding, and a number of infected tsetse were observed from the time they were isolated until their death. Every time that a fly was hungry it was made to probe on to three consecutive slides before it was fed on a rat, and the number of trypanosomes extruded was counted. (In 232 tests every rat except one was infected.) For convenience, probes on three slides, occupying a period of about one and a half minutes, were taken as a 'test.' The test was repeated on each day that the fly was hungry. Table V shows the results of such a consecutive series of tests on a number of flies, from the date of their isolation until their death.

Besides the metacyclic trypanosomes present, there were usually other forms (*crithidia*, *proventricular*, rounded, etc.). Flies differed from one another in the proportion of these forms present; they might constitute up to 50 per cent. of the total number, but the majority of flies had only a small proportion (up to 4 per cent.). Very occasionally it was found that proventricular forms were extruded in very great numbers, forming such a tangled mass that it was impracticable to count them.

Even if this three-slide test did not represent the actual number of trypanosomes extruded when a fly fed, it did allow of a comparison between various flies, and of the behaviour of a single fly on different days.

A statistical analysis of Table V showed that the number of trypanosomes ejected was low and more constant in the first half of a fly's life, and high but more variable in the second. In other words, as the fly aged it had a better, though more variable, chance of inoculating a large dose of trypanosomes on feeding.

Comparing the results of these probe-tests with flies from the various lines and the number of men actually infected, there appeared to be a parallel between the number of trypanosomes extruded in the test and the infectivity of the lines to man. Table V was examined to see what dose of inoculated metacyclic trypanosomes would be the limit, above or below which there would be a similar proportion of infections or non-infections. From this scrutiny it appeared as if a dose of about 350 metacyclic trypanosomes would be required to infect man.

An attempt was then made by one of us (H. F.) to confirm this experimentally. A thin film of egg-albumen was smeared over the counting-chamber of a Turk haemocytometer slide and allowed to dry. A small drop of fluid, consisting of equal parts of sheep serum and Ringer-glucose solution (the solution described by Yorke, Adams and Murgatroyd, 1930), was placed on the slide, and the fly was induced to probe into the fluid. After some seconds the slide was removed, the cover-slip put on, and the total number of trypanosomes in the drop counted. The cover-slip was then removed and the whole drop sucked up into a tuberculin syringe fitted with a fine needle; drops of fluid of the above composition were repeatedly placed on the slide and the cover-slip, and were sucked up until the syringe was full. The contents of the syringe were immediately inoculated subcutaneously into the arm of a volunteer who was waiting, the needle being left in place, the syringe disconnected and refilled with fluid, which was also inoculated so as to wash out any trypanosomes in the barrel.

As a control to the human inoculations, a drop in which only 14 trypanosomes were

seen was sucked up and inoculated into a rat. The rat was infected, with an incubation-period of eight days. A second rat was inoculated with seven trypanosomes, and was infected, with an incubation-period of seven days. As the incubation-periods of rats bitten by flies were usually 5-8 days, the technique was shown to be effective even when only a few trypanosomes were present. It is admitted that there may have been a discrepancy between the number of trypanosomes counted and those inoculated, for, when there was a large number of actively motile forms, the count might originally have been on the low side. The most serious error was the inability to differentiate crithidial forms in the fresh preparation (proventricular forms were sometimes seen and not counted), so that the estimated number of metacyclic trypanosomes inoculated might be unduly high. Table VI gives the results of these inoculations.

Of the 19 men who were inoculated, 11 men were not infected with doses of 88, 111, 128, 143, 146, 177, 188, 206, 220, 308 and 362 metacyclic trypanosomes; four men were infected by 170 (volunteer 12), 284, 389 and 455 (volunteer 168) metacyclic trypanosomes;

TABLE

The total number of metacyclic trypanosomes extruded by *G. morsitans* when probing

Sheep host										
GA. 10	GA. 33	GA. 36	GA. 9	GA. 27	GA. 30	GA. 78	FU. 35	FU. 18	FU. 37	FX. 19
232	125	516	648	48	276	356	14	11	532	32
237	1,008	845	908	9	D	D	D	62	D	29
127	12	358	15+	17	—	—	—	D	—	26
232	527	1,372	D	136	—	—	—	—	—	45
307×	468	583	—	118	—	—	—	—	—	70
157	34+	758	—	108	—	—	—	—	—	153
112	1,637	D	—	495	—	—	—	—	—	18
159	149	—	—	D	—	—	—	—	—	45
842	38	—	—	—	—	—	—	—	—	D
87	39	—	—	—	—	—	—	—	—	—
73	163	—	—	—	—	—	—	—	—	—
367	334	—	—	—	—	—	—	—	—	—
901	341	—	—	—	—	—	—	—	—	—
2,475	337	—	—	—	—	—	—	—	—	—
1,942	79	—	—	—	—	—	—	—	—	—
84	173	—	—	—	—	—	—	—	—	—
1,177	326	—	—	—	—	—	—	—	—	—
2,371	770	—	—	—	—	—	—	—	—	—
3,779	55	—	—	—	—	—	—	—	—	—
3,775	230	—	—	—	—	—	—	—	—	—
335	264	—	—	—	—	—	—	—	—	—
D	91	—	—	—	—	—	—	—	—	—
—	D	—	—	—	—	—	—	—	—	—

D = Fly died.

+ = Flies would only probe on one slide.

and four men were not infected with doses of 680, 800, 833 and 1,067 (volunteer 69) metacyclic trypanosomes.

The high resistance of volunteer 69 has already been referred to. Volunteer 168 was not infected in March, 1943, by the bite of fly II. 54 from monkey 441. In April he was inoculated by syringe with 455 metacyclic trypanosomes from fly IP. 37 and was infected, with an incubation-period of 12 days. This appeared definite evidence that fly II. 54 could not have inoculated a sufficient dose of metacyclic trypanosomes when it bit him a month earlier.

Fly IP. 37 was infected from Thomson's gazelle 39, the same host which infected fly IO. 21. This latter fly on isolation extruded only seven trypanosomes on a three-slide test, and failed to infect the first man bitten, infected the second and third (in each case with no reaction at the site of the bite), and failed to infect the remaining seven men, six of them being bitten a second time. Interspersed with the rebites, a number of probe-tests were done, which gave trypanosome counts of 120, 234, 39, 18, 68, 32, 9, 18 and 6

V

on to three successive albumen-covered slides, on consecutive hunger days

FS. 20	Thomson's gazelle host						Monkey host				Man host
	GE. 14	GE. 33	GF. 36	GF. 2	GF. 30	GF. 42	FN. 22	FT. 28	GN. 16	GN. 56	
11	653	252	41	341	529	549	75	12	26	143	6
170	215	1,096	230	291	2,029	236	418	6	58	98	34
173	2,218	859	83	138	1,846	492	258	25	112	55	6
1,162	720	202	1,062	1,155	5,520	D	304	43	46	1†	322
5,814	603	1,740	1,130	442	1,755	—	143	D	296	143	28 ×
2,651	2,534	728	4,279	844	1,865	—	641	—	379	126	55
1,243	2,959	3,468	D	944	928	—	1,525	—	339	386	446
D	3,375	3,495	—	260	5,214	—	D	—	503	929	197
—	2,720	332	—	2,520	5,330	—	—	—	513	841	592
—	1,346	418	—	D	D	—	—	—	339	D	534
—	20	1,095	—	—	—	—	—	—	229	—	127
—	996	D	—	—	—	—	—	—	D	—	129
—	D	—	—	—	—	—	—	—	—	—	549
—	—	—	—	—	—	—	—	—	—	—	345 +
—	—	—	—	—	—	—	—	—	—	—	278 ×
—	—	—	—	—	—	—	—	—	—	—	D
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—

× = Flies would only probe on two slides.

† = Copious salivation on all three slides; single trypanosome extruded on first slide only.

(some of these counts are recorded in Table IV). The fly also fed on 12 rats, all of which were infected, with incubation-periods of seven days for the earlier rats and more than 35 days and 43 days for the last two rats. The lightness of the trypanosome infection of fly IO. 21 is unique in our experience, and on our hypothesis, save for a short period at the start, it was incapable of extruding an infective dose of metacyclic trypanosomes. Comparing fly IO. 21 with fly IP. 37, from which metacyclic trypanosomes for inoculation were easily obtained, there is no need to postulate a differential change in the 'quality' of the strain in this one test animal, Thomson's gazelle 39, to account for the failure of fly IO. 21 to infect man.

It appears to be established that man *does* require a minimum infective dose of metacyclic trypanosomes, and this would seem to be 300-450 trypanosomes for the average man; but those men who have been shown to have a definite resistance would require a much higher dose.

Assuming that a dose of 300 trypanosomes is the smallest which will infect the *average* man, then a re-examination of Table V furnishes the explanation of a number of previously anomalous results in the tests on volunteers. Some flies were heavily infected from the beginning (GA. 36 and GF. 30); other flies were lightly infected and remained so for life (FX. 19); while others again were lightly infected at first, but the numbers extruded increased with age (GA. 27, GF. 36 and FN. 22). It also brings out the point that even a heavily infected fly had periods when only a few trypanosomes were

TABLE
The results of inoculating a known number

Volunteer no.	Date bitten	Previous history of volunteer		Result	
		By fly	Fly infected from	Arm-puncture positive	Blood positive
104	14.3.41	ER. 41	Monkey 261	10	14
89	17.2.41	ER. 36		7	11
70	21.2.41	EO. 10	T.G. 17	7	11
107	21.3.41	ER. 41	Monkey 261	8	9
39	11.8.36	CT. 11	Steinbuck 1	8	9
115	10.4.41	EY. 59	Sheep 289	—	—
	30.4.41			8	11
124	14.4.41	FA. 58	Sheep M2B	7	9
K.	—	—	—		
12	7.2.38	CC. 37	Sheep 221	N.R.	9
117	19.4.41	EY. 59	Sheep 289	7	8
113	14.4.41	EY. 12	"	—	—
	30.4.41			8	9
17	18.2.38	CD. 38	Dik-dik 52	—	+
	13.2.41	—	Inoc. 50 blood trypanosomes	—	—
168	6.3.43	II. 54	Monkey 441	—	—
49	2.3.40	DU. 68	Reedbuck 11	7	8
	13.12.41	—	Inoc. 50 blood trypanosomes	—	—
99	20.2.41	ER. 41	Monkey 261	—	—
	5.3.41	ER. 36	"	9	14
90	20.2.41	"	"	7	11
88	14.2.41	"	"	—	—
	27.2.41	"	"	9	11
69	17.2.41	EO. 10	T.G. 15	—	—
	3.3.41	ES. 12	T.G. 17	—	—
	21.3.41	ER. 36	Monkey 261	—	—
	8.4.41	—	Inoc. 102,000 blood trypanosomes	8	10

N.R. = No reaction at the site of the infecting bite.

ejected (see flies GA. 10, GA. 33 and GF. 2). This explains why two flies from the same isolation have not the same power to infect man; and it also explains the phases of infectivity and non-infectivity seen in the volunteer tests (see Appendix D, flies ER. 36, ER. 41, EY. 12, EY. 59, FA. 57, GI. 12, GN. 58, GJ. 1 and IJ. 49), or the fact that only alternate bites might be infective (see Appendix D, flies EC. 5, EW. 54, GM. 28 and IH. 14).

We would suggest the following explanation of the varying infectivity to man of this strain of *T. rhodesiense*:

1. There seems to be periods when the strain is easily transmissible, and when all the positive flies are highly infected with trypanosomes. With such highly infected flies the dose of metacyclic trypanosomes inoculated is usually large, and all, or the majority of, men fed upon are infected.

2. While the strain may still be easily transmissible, the trypanosomes do not multiply at the same rate in the salivary glands of each fly. Some flies may be heavily infected, while others from the same isolation are only moderately infected. With such moderately infected flies, man's resistance comes into play, for a dose of 300 metacyclic trypanosomes, which may be infective to A, is not sufficient for B, who may require 1,000 or more.

3. When the ability of the trypanosomes to multiply in the salivary glands is low (and this usually coincides with low transmissibility in normal flies, under ordinary conditions), the infection of the salivary glands may be so light that only rarely does a fly inoculate more than the minimum infective dose of metacyclic trypanosomes.

VI of metacyclic trypanosomes into volunteers

Experimental inoculation					
Date of inoculation	Trypanosomes from fly	Fly infected from	Trypanosomes counted on slides	Result	
				Arm-puncture positive	Blood positive
30.3.42	GO. 4	T.G. 30 ex sheep	111	—	—
2.4.42	"	" "	206	—	—
3.4.42	"	" "	88	—	—
6.4.42	"	" "	800	—	—
6.4.42	"	" "	220	—	—
8.4.42	GN. 56	Monkey 332	128	—	—
9.4.42	GO. 4	T.G. 30 ex sheep	389	11	16
9.4.42	"	" "	284	8	11
12.4.42	"	" "	170	9	11
24.4.42	GV. 4	T.G. 31	143	—	—
27.4.42	GV. 19	"	308	—	—
8.4.43	IP. 37	T.G. 39	146	—	—
4.5.43	IN. 11	Sheep 327	833	—	—
9.4.43	IP. 37	T.G. 39	455	11	12
14.4.43	"	"	177	—	—
20.4.43	IN. 11	Sheep 327	680	—	—
25.4.43	"	"	188	—	—
28.4.43	"	"	362	—	—
29.4.43	IJ. 49	Monkey 441	1,067	—	—

TABLE VII
The incubation-period of the disease in 206 volunteers who were infected

Incubation-period, in days	7	8	9	10	11	12	13	14	15	16	17	18	20	21	25	27	29	30	Reaction positive, blood negative when treated	No record 1936-38	Total
Cases with arm- reaction ...	7	20	36	34	28	19	4	8	3	1	1	1	1	1	1	-	1	1	6	16	189
Cases with no arm- reaction ...			6	4	1	2		2					1			1*					17

* The blood of volunteer 128a was inoculated into a rat on the 27th day and he was treated ; the rat was infected, with an incubation-period of 28 days.

TABLE VIII

The monthly transmissibility-rate of the sheep line for the years 1936-42 inclusive, and the mean maximum screen temperature averaged over the same period

	Jan.	Feb.	March	April	May	June	July	August	Sept.	Oct.	Nov.	Dec.
Percentage of flies infected ...	3.3	4.2	1.7	2.1	1.5	2.2	4.0	3.3	8.4	3.7	3.6	2.5
Mean maximum temperature ...	23.6° C.	23.7° C.	23.9° C.	23.5° C.	23.4° C.	22.8° C.	22.2° C.	23.0° C.	24.9° C.	26.0° C.	25.2° C.	23.9° C.

4. The virulence of the strain may also be a factor, for the minimum infective dose of a virulent line may be smaller than that of a less virulent one.

On this hypothesis, the ability of the fly to inoculate an infective dose of metacyclic trypanosomes is the resultant of at least two factors: (a) the power of the trypanosome to invade the salivary glands (i.e., the transmissibility of the strain), and (b) the ability of the trypanosome to multiply in the glands.

Trypanosomes may invade the salivary glands of only a few flies, but they may multiply markedly, giving a high dose on feeding. On the other hand, trypanosomes may invade the salivary glands of a large number of flies, giving a high transmissibility-rate, but the infection of the glands may be a light one, and the dose of metacyclic trypanosomes inoculated small and non-infective to man.

Of the 183 cases whose incubation-periods were known definitely, the blood of 144 cases (78.6 per cent.) was positive between the 7th and the 12th day after being bitten, and the blood of 159 cases (86.9 per cent.) was positive between the 7th and the 15th day.

Manson taught that an indurated, inflammatory lesion developed at the site of an infective tsetse bite. Graf (1929) was apparently the first to show that by the puncture of this lesion trypanosomes could be demonstrated some days before they were found in the blood-stream. Subsequently (1937) he stated that this trypanosome chancre represented a tissue reaction to the injected trypanosomes, and he considered that a high virulence of the parasite and a low resistance of the host favoured the development of the chancre.

Of the 17 men who had no reaction at the site of the infecting bite, four men (volunteers 93a, 128a, 141a and 158a) were infected on a rebite by the same fly that had failed to infect them at first; and it has been shown that, as a fly aged, there was a better chance of its inoculating a higher dose of trypanosomes on feeding. Volunteer 93a was the only infection in a long series of bites and rebites by fly ER. 36 from monkey 261. Volunteer 128a was rebitten by fly GI. 2 infected from sheep 302, and, as he showed neither signs nor symptoms on the 27th day, 1 c.cm. of undiluted blood was inoculated into a rat, and he was treated; the rat was infected, with an incubation-period of 28 days.

Of the 13 men who had no reaction when they were infected on a first feed, the following points should be noted. Volunteer 91 was infected by fly ER. 36 from monkey 261; and volunteer 138 was infected by fly GM. 28 from Thomson's gazelle 30 ex sheep; and both these flies, when they rebite, infected men (volunteers 93a and 141a) without a local reaction being produced. Volunteers 208 and 209 were the only two men infected by fly IO. 21 from Thomson's gazelle 39 in a long series of bites and rebites, and it has already been noted how few trypanosomes fly IO. 21 extruded on successive probe-tests (p. 287). Fly MU. 20 from Thomson's gazelle 62 infected only three men out of 14 fed upon, and two of these men (volunteers 251 and 254) had no reactions. Fly PZ. 21 from reedbuck 17 only infected three men out of eight fed upon; two of these men (volunteers 306 and 307) had no reaction, while the third man had an incubation-period of 18 days. Fly QB. 45 from monkey 637 probed into the arm of volunteer 314 for two minutes, but did not appear to obtain a feed of blood. The man was infected, with an incubation-period of nine days, with no reaction at the site of the probe. In a subsequent section (p. 295) it will be shown that the virulence of the strain in the Thomson's gazelle, monkey and sheep lines was high at the time that these patients were infected *without* developing local reactions.

There was no statistically significant difference in incidence of no reactions on first bites and on rebites.

The history of volunteer 181 is of interest. He was bitten by fly IK. 26 from sheep 327, and on the 10th day a small nodule about 1/16 in. in diameter was palpable in and under the skin. Puncture of this nodule yielded trypanosomes. On the 11th day his skin was perfectly smooth, no nodule being palpable, and trypanosomes were present in his blood (3 in 74 fields).

If it is accepted that man requires a minimum infecting dose of trypanosomes, and if it is accepted that men vary in their resistance, then the infective dose of metacyclic trypanosomes will vary from man to man. From the evidence presented, it appears to one of us (H. F.) that no reaction develops at the site of the bite when the fly inoculates a *minimal* infecting dose of metacyclic trypanosomes; but that, when more than this dose is inoculated by a fly, then a 'trypanosome chancre' appears. The virulence of the parasites and the resistance of the host, as postulated by Graf, merely control what would be a non-infective, minimal infecting or excessive dose of parasites. If this conclusion is correct, then the fact that volunteer 12 had no reaction when he was bitten by fly CC. 37 from sheep 221, but did develop a reaction when he was inoculated by syringe with 170 metacyclic trypanosomes, shows that his resistance must have been low. A comparable case of low resistance is volunteer 334, referred to on page 304.

V. FACTORS INFLUENCING THE TRANSMISSIBILITY AND DOSAGE OF THE STRAIN

A considerable amount of work has been recorded in the literature on the factors which influence the transmissibility of the polymorphic trypanosomes (i.e., their ability to invade the salivary glands); but little is known about the factors which influence the multiplication of trypanosomes in the salivary glands.

1. *The Trypanosome.* Duke (1933a) stated that light infections of a gland were commoner than was realized, and he recorded instances of unilateral gland infections. The light salivary gland infections produced by strain Tinde I as compared with strains Tinde II and Tinde III (Duke 1933b) are an example of the differing ability of strains of *T. rhodesiense* to multiply in the glands.

The statistical analysis of the results recorded in Table II showed that there was a correlation between transmissibility and infectivity of the three lines maintained in sheep, Thomson's gazelle and monkey. There were statistically significant decreases in infectivity in both the sheep and the Thomson's gazelle lines (fig. 1), but since each occurred in a different year, namely, 1940 and 1944 respectively, no external factor, such as temperature, could have been the cause.

As infectivity is controlled by dosage, and as dosage is controlled by the power of the trypanosome to multiply in the salivary glands of a fly, the variation in infectivity of the sheep and Thomson's gazelle lines over a period of years shows a fluctuation in the ability of the trypanosome to establish itself in the salivary glands, a fluctuation which is made evident by maintenance of the strains for a number of years in a single species of host.

2. *The Host.* Robertson (1912), Van Hoof, Henrard and Peel (1937b) and Corson (1935, 1936b, 1938c) have shown that 'infected buck,' the monkey *Cercopithecus galeritus agilis*, and the southern reedbuck, *Redunca arundinum* Boddaert (known locally as a

'monge'), give increased transmissibility-rates of the polymorphic trypanosomes compared with other hosts.

In our experience, higher transmissibility-rates have been secured by maintaining the strain in the Bohor reedbuck, *Redunca redunca* Pall., than in sheep, Thomson's gazelle or monkey. But an analysis of our records showed that, where the strain was transferred to another species of host, transmissibility and dosage were both increased or decreased, i.e., there was again no dissociation of the two factors.

3. *Temperature and the Fly.* Kinghorn and Yorke (1913*b*) first showed that there was a critical temperature below which *T. rhodesiense* did not invade the salivary glands of *G. morsitans*, although development had proceeded in the fly as far as the proventricular forms. They also showed the effect of the difference of temperature of the hot and cold seasons on the percentage of wild flies infected. Chorley (1929) also noted the same seasonal rise in the hot weather of *G. morsitans* infected with *T. congolense* and *T. vivax*.

The transmission-data for the sheep line for the years 1936-42 were averaged month by month, and it was found that there was a marked monthly variation. Transmissibility varied between 1.5 per cent. of flies positive in May and 8.4 per cent. in September; and there was a significant correlation between the monthly percentage of flies infected and the mean maximum screen temperature, taken at Shinyanga 30 miles away, and averaged over the same period. The data are given in Table VIII (p. 290).

Over and above the monthly variation, we have found that there were periods of the year when it was difficult to secure a cyclical transmission in the various lines. Broadly speaking, these difficult periods were: sheep line, December-February, during the early rains; Thomson's gazelle line, December-February, during the early rains, and April, during the heavy rains; monkey line, March-May, during the heavy rains; dik-dik, July and August, the early dry season. Corson (1939*b*) reported four failures to infect tsetse-flies from dik-dik 50, and said that the most probable explanation seemed to be that the failure was due to the blood of this animal being unsuited for the development of the trypanosome in the flies. These failures to transmit occurred in July-August, 1937. Successful transmissions were subsequently obtained from other dik-dik, except for two failures in April and August, 1938, respectively. The more probable explanation seems to be that transmissions were attempted when temperature conditions were unsuitable, for it was the coolest time of the year, rather than that the blood of a single animal had had an adverse effect on the trypanosomes.

No tests of the infectivity of the sheep line were done in the months of very high transmissibility, so it is impossible to say if temperature had also influenced the multiplication of the trypanosomes in the glands.

Lloyd (1930) and Taylor (1932) showed that, by incubating *G. tachinoides* during the period of feeding on the host and after, a very much greater transmissibility-rate was obtained; but here again there was no evidence as to what influence temperature may have had on dosage.

Some new work (Burt, 1946*a*) helps to elucidate this point. Pupae for this laboratory are collected in Kondoia-Irangi District, and are posted in batches of 500-1,000 every week or fortnight, as the work requires. On receipt the pupae are placed in wide-mouthed bottles closed with mosquito-gauze, and the bottles are placed mouth downwards on wire-mesh trays standing over water. Every morning the flies which have emerged during the previous 24 hours are transferred to Bruce boxes and fed on an infected animal. With

this standard maintenance, under normal laboratory conditions, it was usually found that less than 10 per cent. of the surviving flies had infections of the salivary glands—a point already made by Corson (1935).

In March, 1943, the emergence of flies from pupae was too slow to keep pace with requirements. The bottles with pupae were therefore placed in an incubator kept at approximately 30° C., and the flies which had emerged during the previous 24 hours were transferred to Bruce boxes, fed on an infected animal, and thereafter kept under standard laboratory conditions. From March, 1943, transmission-rates of 20 per cent. in the sheep line, 22.6 per cent. in the Thomson's gazelle line, and 34.0 per cent. in the monkey line were being obtained, all percentages calculated on the flies surviving to be isolated. These high transmission-rates were difficult to explain, as temperature conditions in the laboratory at that time were not unusually high; but, on re-examination of the records, it was found that the high transmissibility had occurred in batches of flies from incubated pupae. And not only did flies from incubated pupae give high transmission-rates, but in not a single experiment had there been a failure to secure a transmission.

Ignoring the effect of the various host animals, the results up to date can be summarized as follows:

	Flies from normal pupae	Flies from incubated pupae
Total no. of transmission-experiments ...	238	97
No. of experiments where transmission failed...	54	Nil
" flies surviving to be isolated ...	7,127	3,352
" " infected ...	296	408
Percentage of flies infected ...	4.2	12.2

Seven experiments were therefore done in parallel: (A) with flies from normal untreated pupae, and (B) with flies which had emerged from incubated pupae. Boxes of (A) and (B) were fed at the same time on the same host. Two of the experiments with flies (A) failed to give a transmission, and there were 2.3 per cent. of infected flies in the remainder; while with flies (B) transmissions were secured in every case, and there were 10 per cent. infected flies.

Trypanosome counts were done from a number of infected flies which had emerged from incubated pupae, and a few examples are given in Table IX.

TABLE IX

The number of metacyclic trypanosomes extruded on a three-slide test on various days by infected flies which had emerged from incubated pupae

Year	Host	Transmission-rate of isolation	Fly	No. of metacyclic trypanosomes counted on various days
1943	T.G. 39	18.2 per cent.	IO. 10	165; 1,299; 11,343
			IO. 21	7; 120; 234; 39; 11; 40; 6; 5; 1; 4
	T.G. 51	21.1 "	KB. 42	11,574; 2,355; 1,091; 3,107
	"	18.0 "	KQ. 16	277; 78; 348
			KQ. 24	64
			KQ. 55	644; 694; 769
	Monkey 483	26.7 "	KN. 12	17; 56; 25; 6; 207

The counts of 11,343 metacyclic trypanosomes extruded by fly IO. 10, and of 11,574 extruded by fly KB. 42, are the highest yet recorded in these probe-tests.

Hoare (1931), discussing the transmissibility of *T. grayi* and *T. gambiense* by *G. palpalis*, came to the conclusion that the rôle of the insect host was far from being passive, and was more consistent with the view that among the Glossina certain individuals were immune; while Duke (1933a) thought that certain individuals among the tsetse population were specially fitted to act as intermediate hosts of the pathogenic trypanosomes.

On the present evidence, the effect of incubating tsetse pupae appears to be that flies which would normally be infected with trypanosomes have a heavier infection than usual, while other flies are infected which would normally be immune. These latter flies often have a very light infection of the salivary glands, enough to raise the transmissibility-rate but not enough to inoculate an infective dose to man. In other words, by the incubation of pupae one has dissociated transmissibility and dosage. This dissociation must be due to some alteration in the physiology of the fly; but the exact mechanism behind it remains to be investigated.

Burt (1946a) also found that the trypanosome-cycle in flies from incubated pupae was significantly shorter than in flies from normal pupae; and he found that a significantly greater number of flies from incubated pupae were alive to be isolated than flies from normal pupae.

It may be that the ultimate cause of the higher infection of flies in the dry season is due to the higher temperatures to which the pupae have been exposed, before the flies emerge.

Should this prove to be the case, it would have great practical importance. For by inefficient methods of bush-clearing it may be possible markedly to reduce the number of flies present, but the pupae of those that remain might be exposed to higher temperatures than would normally have been the case. The flies emerging might then have a higher transmissibility-rate, and might possibly inoculate a higher dose of trypanosomes than they might normally have done.

VI. THE VIRULENCE OF THE STRAIN

The virulence of the strain, as judged by the length of life in the infected animals, in the various lines was examined, the data being given in Appendices A and B.

For the years 1934-38 the length of life of sheep was taken as the period elapsing between transferring the fly-boxes from the infected animal to the clean one and the death of the latter. This period therefore included an indefinite number of days during which the cycle was being completed in the flies plus the incubation-period in the new animal. To keep the data comparable, the same procedure was followed in estimating the length of life in sheep in the period 1939-44. The duration of life of monkeys was the number of days between the blood first becoming positive on microscopical examination and death.

The numbers of observations of sheep and monkeys were sufficient for statistical analysis, but this was not the case for the other host animals (Thomson's gazelle, reedbuck, etc.). The flies which had been infected from all animals had, however, been isolated from 1940 onwards, and they were fed on separate clean rats every time they were hungry. In this way there was a large number of rats which had been infected from the different lines. Statistical analysis of the length of life in rats infected from the sheep and monkey

lines showed that there was a close parallel between the length of their lives and the lives of the sheep and monkeys themselves. We therefore considered it justifiable to use the duration of life in rats infected from the other host animals as an index of the virulence of the trypanosome in those hosts. The following results obtained are shown diagrammatically in fig. 3.

The longevity of the sheep themselves was significantly longer in the period 1942-44 than in 1934-37 ($P < .01$). There was no significant difference on comparing year by year; but, if the data were grouped, then it was found that there was a significantly longer life in the period 1938-41 than in 1934-37, and a significantly longer life in the period 1942-44 than in 1938-41. These results show that there has been a gradual and continuous *loss* of virulence of the trypanosome maintained in sheep for the last 10 years.

The length of life of rats infected from the sheep line has shown the same gradual and continuous loss of virulence over the period 1940-45. (Rats in the period 1944-45 lived significantly longer than in 1940-43, but there was no significant difference when the data were examined year by year.)

The longevity of the monkeys showed no significant alteration during 1940-43; but in 1944 they lived for a significantly shorter time than in 1943 ($P < .01$). There was no significant difference in the longevity between 1944 and 1945, but the two years 1944-45 taken together showed a significantly shorter life than 1940-43 ($P < .01$). There was therefore a significant *increase* in the virulence of the trypanosome in the monkey line between 1943 and 1944.

The examination of the longevity of rats infected from the monkey line showed that there was a significantly shorter life in 1943 than in 1940-42 ($P < .01$), and that the life of rats was again significantly shorter in 1944 than in 1943 ($P < .01$). There was no significant difference between the *differences* of 1940-42 versus 1943, and 1943 versus 1944, which suggests that the increase in virulence was gradual over a period of years. A comparison of the rats infected from the monkey line with the monkeys themselves gives the same picture, except that the increase in virulence was apparent in the rats infected from monkeys a year earlier than it was evident in the monkey hosts themselves.

The rats infected from the main Thomson's gazelle line lived a significantly shorter time in 1941 than in 1940 ($P < .05 > .02$); there was no significant difference between the years 1941, 1942 and 1943; but the rats in 1944 lived a significantly shorter time than in 1943 ($P < .01$), with no further alteration in 1945. There was thus an *increase* in virulence of the trypanosome in the Thomson's gazelle line over the period 1940-45.

The loss of virulence, as determined above, of the sheep line, and the increase in virulence of the Thomson's gazelle and monkey lines, were confirmed by comparing the three lines. During 1940 there was no significant difference in the longevity of the rats from the sheep and Thomson's gazelle lines; in 1941 the longevity of the Thomson's gazelle rats was shorter; in 1942 there was no difference—this being probably due to the small number of rats available for examination; but in the years 1943, 1944 and 1945 the Thomson's gazelle rats lived a significantly shorter time than the rats infected from sheep. The monkey line was started from the sheep line in 1940. In the years 1940-42 there was no significant difference between the two lines, and the rats infected from monkeys lived significantly longer than those from Thomson's gazelle. From 1943, when the increase in virulence first became apparent in the rats infected from monkeys, until 1945, these rats now lived a significantly shorter time than the rats from sheep, and there

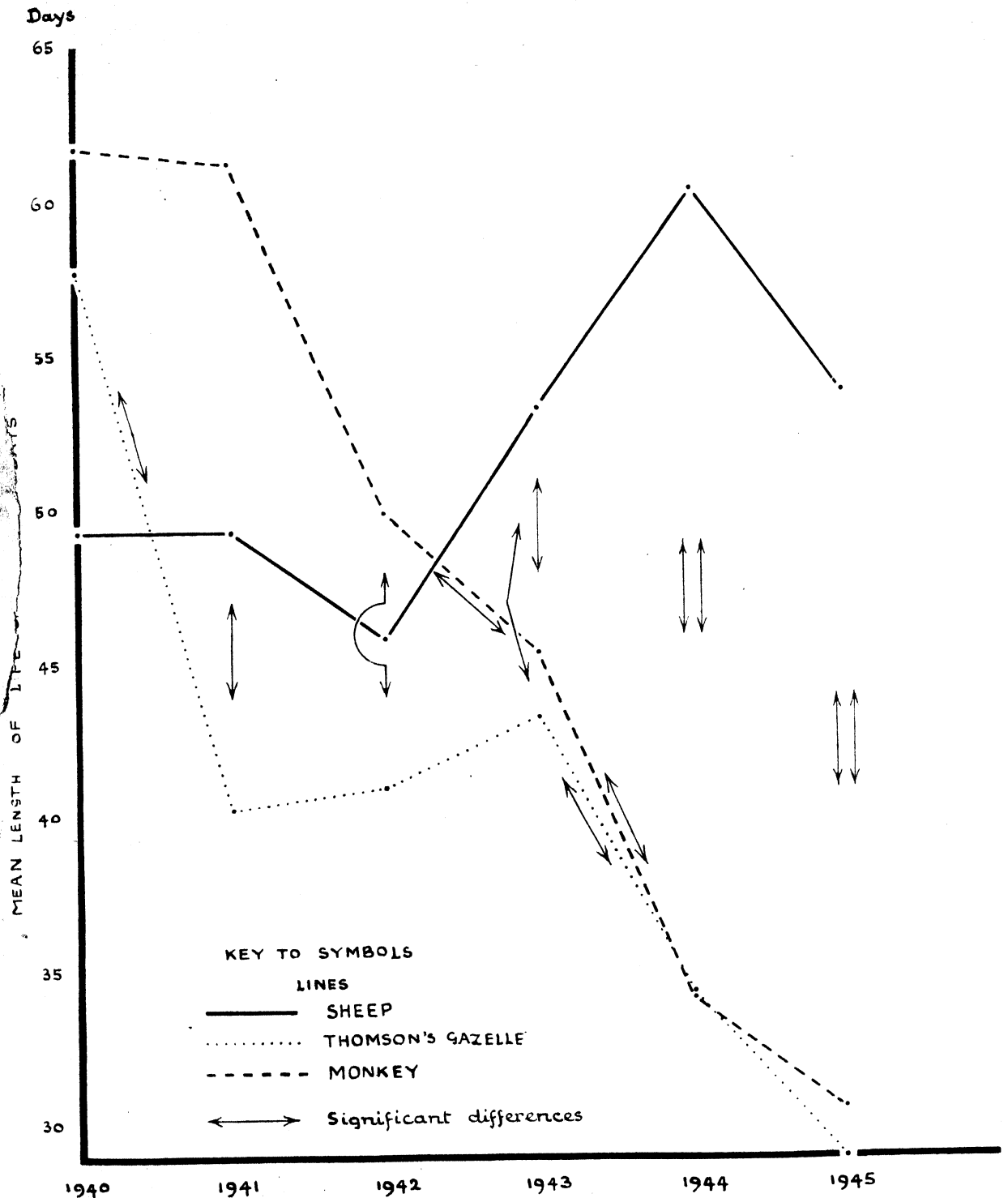


FIG. 3. Diagram illustrating the statistical analysis of virulence as judged by the mean length of life of rats.

was no difference between the rats from monkey and Thomson's gazelle. The virulence of the trypanosome in the monkey line had diverged from that of the sheep line and approximated to that of the Thomson's gazelle line.

When the antelope line was first maintained solely in Thomson's gazelle from 1940 onwards, there was an alteration in virulence, as the rats infected from the latter lived significantly longer than those infected in 1939 from the mixed antelope line ($P < .01$), but the virulence of the trypanosome recovered in time. Maintenance of the trypanosome solely in sheep had produced a progressive loss of virulence; but the virulence was restored by passage through monkeys after a time-lag.

On comparing the data on virulence to rats and infectivity to man (Table I and fig. 1), it will be seen that, while the virulence of the sheep line had progressively decreased, the infectivity to man had dropped to a low point in 1940 and then risen by 1945 until it was very highly infective; but in the monkey and Thomson's gazelle lines the infectivity to man had fallen during recent years, while in these same years the virulence of the lines had increased. This demonstrates that infectivity to man is not bound up with the virulence of the trypanosome, and that virulence and infectivity to man are two separate and independently variable qualities of a trypanosome.

The incubation-period of the disease in men infected from the Thomson's gazelle line (1940-45) and from the monkey line (1941-45) has remained constant. The incubation-periods in men infected from the sheep line were as follows:

1936	...	7, 7 days.
1938	...	9 days.
1940	...	9, 10, 11 days.
1941	...	9, 8, 8, 9, 10, 9, 11, 11 days.
1942	...	9, 12, 10, 10, 9, 10, 9 days.
1943	...	14, 11, 13, 12, 12, 12, 15, 12 days.
1944	...	25, 10, 14, 20, 11, 11, 12 days.
1945	...	12, 10, 15, 14, 10, 11, 11, 12, 9, 9, 12, 13, 10 days.

Statistical analysis shows that in 1943 there was a significantly longer incubation-period than in 1942 ($P < .01$), and that the incubation-period was significantly longer in the years 1943-45 than in 1936-42 ($P < .01$).

Kligler and Rabinowitch (1927) reported that, in guinea-pigs inoculated intraperitoneally with *T. evansi*, the incubation-period was dependent, within broad limits, on the dose of trypanosomes injected. The infectivity of the sheep line to man has not altered significantly over the period 1942-45 (see fig. 1); and if the dose of metacyclic trypanosomes inoculated by a fly is correlated with the transmissibility of a line, as has been argued, Table II gives no evidence of decreased transmissibility of the sheep line during this period; so it would appear as if the prolongation of the incubation-period in man during 1943-45 was actually caused by the decrease in virulence of the sheep trypanosome, which we know to have taken place. The clinical character of the disease in man in 1945 also supports this, for the arm-reactions were smaller and softer than in patients infected from the other two lines, and, of the 13 men infected, four of them had normal temperatures and a further two had temperatures of only 99.2° F. and 99.8° F. when their blood was first microscopically positive.

The number of days taken by the trypanosomes of the sheep, monkey and Thomson's gazelle lines to complete the cycle in the fly is given in Appendix C. Statistical analysis showed that the length of the cycle of the sheep line was significantly longer than the cycle of the Thomson's gazelle line ($P < .01$), there being no significant difference between the sheep and monkey or between the monkey and Thomson's gazelle lines.

VII. THE INFECTION OF RATS FROM MAN

Corson (1938c, 1939a) reported occasions when 1 c.cm. of the undiluted blood of a volunteer who was infected with *T. rhodesiense* (and whose blood showed scanty trypanosomes microscopically) inoculated subcutaneously into each of two rats failed to infect them. An examination of our records showed that in 1940 two mice inoculated with positive blood from volunteer 49 (reedbuck 11, main antelope line) failed to be infected, and that in 1943 five rats inoculated from volunteer 191 (reedbuck 12 ex sheep) were not infected. In 1944 the blood of volunteers infected from the sheep line was used to inoculate rats. Six rats inoculated with positive fluid from the arm-reactions of four men, and nine rats out of 24 which were inoculated with positive blood from five men, failed to be infected. On the other hand, the blood of three men infected from the Thomson's gazelle line infected all of 12 rats inoculated, and the blood of three men infected from the monkey line infected all of nine rats inoculated, while of three rats inoculated with fluid from the arm-reaction one failed to be infected.

These results were so suggestive of an alteration in character of the trypanosome in the sheep line that it was decided to inoculate rats from the arm-reaction and from the blood of every volunteer in 1945 whenever the reaction or the blood was found to be positive. The following technique was used:

1. *Arm-Reaction.* The arm-reaction having been proved to be positive, it was repunctured and a thick blood film was made from the first drop, so as to confirm the presence of trypanosomes. Further drops were then expressed and drawn up into a 1 c.cm. syringe filled with citrated saline and fitted with a short stout needle, until the bloody fluid appeared in the barrel of the syringe. (As the same syringe and needle were always used, approximately the same amount of fluid was taken from each volunteer's arm for inoculation.) The fluid was well mixed with the citrated saline in the syringe, and 0.5 c.cm. of the mixture was inoculated subcutaneously into each of two rats.

2. *The Blood.* Within a couple of hours of the patient's blood having been examined in a stained thick film and found positive, the antecubital vein was punctured and 5 c.cm. of blood was drawn up into a dry syringe. One c.cm. of the undiluted blood was immediately inoculated subcutaneously into each of a number of rats, usually three.

The blood of every rat inoculated was examined *daily* in a fresh film from the 4th to the 30th days inclusive, and thereafter the rats were examined twice weekly or weekly, as pressure of work allowed, until the 60th day, when they were considered to be negative. An incubation-period of 39-43 days therefore means that on the 38th day no trypanosomes were present in a fresh film, but that they were found on the 43rd day. The results of all such inoculations of rats from 1940 onwards are shown in Table X.

The fluid from the arm-reaction, and the blood, of the men from the monkey line infected all the 26 rats inoculated. In the Thomson's gazelle line, the fluid from the arm-reaction infected all the 10 rats used, while the blood of four men infected the 16 rats inoculated. The blood of volunteers 283 and 286, however, failed to infect any of the three rats inoculated from either man. The incubation-period in all the above infected rats was 5-12 days. On the other hand, in the sheep line, fluid from the arm-reaction of 12 men infected only six of the 24 rats inoculated (and one of these rats had an incubation-period of 31-37 days), while, of the 44 rats inoculated with blood from 13 men, only 21 were infected (and four rats had incubation-periods of 39-43, 39-43 [volunteer 296], and 32-33 and 44-50 days [volunteer 300]). Of the men infected from reedbuck 17 (ex sheep), the blood of volunteers 306 and 307 infected the rats, but the two rats inoculated from the arm-reaction of volunteer 310, and four out of the six rats inoculated with his blood, were not infected. (One of the infected rats had an incubation-period of 30 days.) The failure to infect rats from the arm-reaction of volunteer 334 is discussed below.

TABLE X
The inoculation of rats from positive arm-reactions, and with positive blood, of men infected with *T. rhodesiense*

Volunteer no.	Infected from	Arm-reaction				Blood			
		Date volunteer bitten	Date positive	Date rats inoculated	Trypano-somes present	Incubation-period of rat	Date positive	Date rats inoculated	Trypano-somes present
36	Dik-dik 53	22.4.38	29.4.38				30.4.38	30.4.38	3/100
49	Reedbuck 11	2.3.40	9.3.40				10.3.40	11.3.40	6/10
191	" 12	7.3.43	16.3.43				5.4.43	5.4.43	3/129
228	Sheep 338	20.4.44	6.5.44	6.5.44	1/60	0	4.5.44	4.5.44	1/200
230	"	24.4.44	3.5.44	4.5.44	2/90	0			0, 19/20, 19/20, 26/28, 26/28
231	"	26.4.44	6.5.44	6.5.44	2/67	0			
232	"	29.4.44	10.5.44	10.5.44	1/150	0			
234	"	8.5.44	10.5.44	12.5.44	2/87	0	19.5.44	19.5.44	1/200
236	"	12.5.44			3/24	0	19.5.44	20.5.44	2/200
237	"	15.5.44					23.5.44	23.5.44	3/170
243	T.G. 62	10.5.44					27.5.44	29.5.44	3/40
251	"	7.6.44	N.R.				19.5.44	20.5.44	4/47
254	"	20.6.44	N.R.				16.6.44	16.6.44	1/20
265	Monkey 593	14.6.44					30.6.44	30.6.44	3/43
267	"	19.6.44	29.6.44	29.6.44	3/32	+	24.6.44	26.6.44	3/60
269	Monkey 603	2.8.44	12.8.44	14.8.44	3/49	+	15.8.44	15.8.44	2/162
276	"	13.8.44			4/48	0	24.8.44	24.8.44	2/25
314	Monkey 637	10.4.45	N.R.				19.4.45	20.4.45	3/48
316	"	13.4.45	20.4.45	21.4.45	3/52	9, 20	23.4.45	24.4.45	3/17
318	"	9.4.45	17.4.45	17.4.45	4/36	10, 13	20.4.45	20.4.45	3/86
322	"	26.4.45	3.5.45	3.5.45	2/173	8, 8	3.5.45	3.5.45	1/200
323	"	30.4.45	6.5.45	6.5.45	3/34	7, 8	7.5.45	9.5.45	3/35
									3/30

0, 0, (Corson, 1939a)
0, 0
0, 0, 0, 0, 0

0, 0, 0, 11/13
10/12, 10/12, 10/12,
12/16, 12/16
0, 0, +, +, +
0, 0, 0, +, +
10/11, 10/11, 10/11,
10/11, 10/11
8/12, 8/12, 8/12,
8/12, 8/17
6, 6
+, +

12/13, 12/13
8, 8, 9/11, 9/11,
9/11

6, 6, 7
6, 6, 6
6, 6, 6
5, 6, 6
5, 6, 6
5, 5, 5

	T.G. 75	5.3.45	15.3.45	16.3.45	4/63	7, 11	13.3.45	13.3.45	3/96	8, 8, 8, 9, 9
280	"	6.3.45	15.3.45	16.3.45	4/63	7, 11	13.3.45	13.3.45	3/3	5, 6, 6, 6, 7
281	"	9.3.45	17.3.45	18.3.45	2/319	7, 15	18.3.45	18.3.45	3/42	0, 0, 0
283	"	12.3.45	18.3.45	19.3.45	3/67	7, 7	20.3.45	20.3.45	3/34	6, 6, 9
284	"	13.3.45	21.3.45	21.3.45	5/49	9, 9	23.3.45	24.3.45	3/61	8, 9, 12
285	"	15.3.45	22.3.45	23.3.45	3/30	7, 7	25.3.45	25.3.45	2/252	0, 0, 0
286	Reedbuck 17	10.4.45	N.R.				22.4.45	22.4.45	3/36	13, 14, 15
306	"	13.4.45	N.R.				23.4.45	23.4.45	3/12	8, 9, 10
307	"	22.4.45	7.5.45	7.5.45	3/164	0, 0	10.5.45	10.5.45	1/200	0, 0, 0
310									3/150	0, 21, 30
289	Sheep 346	14.3.45	23.3.45	23.3.45	2/74	0, 11	26.3.45	12.5.45	3/68	0, 0, 0
290	"	16.3.45	23.3.45	23.3.45	2/200	8, 21	26.3.45	27.3.45	3/121	0, 0, 9
291	"	18.3.45	28.3.45	28.3.45	1/300	0, 0	2.4.45	6.4.45	2/200	0, 0, 0
292	"	19.3.45	29.3.45	29.3.45	2/250	0, 0	2.4.45	4.4.45	3/244	0, 0
293	"	21.3.45	31.3.45	31.3.45	2/41	0, 0	31.3.45	2.4.45	3/25	8, 15, 16
294	"	24.3.45	2.4.45	3.4.45	1/200	0, 0	4.4.45	5.4.45	2/265	0, 17, 20
295	"	28.3.45	4.4.45	5.4.45	1/200	11, 14	8.4.45	9.4.45	3/9	7, 7, 12
296	"	29.3.45	9.4.45	10.4.45	3/17	0, 0	10.4.45	12.4.45	3/231	9, 39/43, 39/43
297	"	1.4.45	7.4.45	7.4.45	1/80	0, 0	10.4.45	10.4.45	2/198	0, 0, 0
298	"	5.4.45	14.4.45	14.4.45	4/115	0, 0	14.4.45	24.4.45	3/147	0, 0, 0
299	Sheep 351	30.4.45					14.5.45	14.5.45	1/200	0, 0, 0
300	"	2.5.45	13.5.45	14.5.45	3/94	0, 0	15.5.45	15.5.45	3/136	0, 32/33, 44/50
302	"	14.5.45		15.5.45	3/45	0, 0		16.5.45	3/33	0, 14, 30
326	Volunteer 280	20.4.45	28.4.45	28.4.45	3/67	11, 14	24.5.45	24.5.45	3/78	16, 17, 18
327	"	22.4.45	30.4.45	30.4.45	3/37	9, 9	30.4.45	25.5.45	3/255	0, 18, 18
333	"	4.5.45					30.4.45	30.4.45	2/200	8, 9, 12
334	"	9.5.45	19.5.45	19.5.45	2/200	0, 0	13.5.45	1.5.45	3/61	7, 7, 8
				20.5.45	3/184	0, 0		1.5.45	4/10	4, 4, 4
				22.5.45	1/200	0, 0		14.5.45	3/50	4, 6, 12
				24.5.45	3/42	0, 21				
				25.5.45	2/200	0, 0				
				7.6.45	1/250	0, 9				
				8.6.45	3/112	0, 10				
				9.6.45	3/72	0, 8				
				10.6.45	3/49	0, 16	15.6.45	15.6.45	3/57	8, 11, 13

'Trypanosomes present 6/10' means that six trypanosomes were found in 10 fields of a stained thick film, using an oil-immersion lens.
 N.R. = No reaction at the site of the infecting bite.
 + = Rat infected.
 0 = Rat not infected.

Statistically, the number of failures to infect rats was significantly greater in the sheep line than in the Thomson's gazelle line ($P < .02 > .01$) or in the monkey line ($P < .01$); and, comparing the results from these three lines (Table X), it will be seen that the infection of rats was not dependent upon the number of trypanosomes present in the inoculum, although this might influence the incubation-period of the disease. The character of the disease in the rats infected from men who were themselves infected from sheep was also different from the other two lines. The incubation-period was longer, after several weeks' observation trypanosomes were often still scanty in the rat's blood, the disease was chronic and relapsing in character, and posterior-nuclear forms were scarce and often only found in small numbers after several examinations. This picture, and the large number of failures to infect other rats, show that there must have been some alteration in the trypanosome itself after prolonged maintenance in sheep.

As there were so many failures to infect rats from the sheep line with blood containing trypanosomes, the inoculation of rats with blood which is still microscopically negative, in order to diagnose early or 'cryptic' infections, can no longer be regarded as a reliable test.

Clean laboratory-bred flies were fed on three men infected from the Thomson's gazelle line and on one man infected from the monkey line, and infected flies were isolated. The flies fed on a number of rats; and, when the longevity of these rats was compared with the longevity of the rats inoculated with blood from the same men, it was found that the fly-infected rats lived a significantly shorter time than the inoculated rats ($P < .01$). In a review of the data from all animals, it was again found that the life of rats infected by flies was significantly shorter than that of rats infected by blood inoculation from the same hosts from which the flies themselves were infected. This means that, in rats infected by the syringe inoculation of blood, the virulence of the strain had been immediately decreased, at least for the first passage; but since, as in the rats infected by syringe, fly-infected rats of the sheep line still lived significantly longer than those from the monkey and Thomson's gazelle lines, the decrease in virulence caused by syringe passage must have been of the same order in all three lines.

VIII. ATYPICAL INFECTIONS OF MAN WITH *T. RHODESIENSE*

During the course of the volunteer tests a number of atypical infections of man with this strain of *T. rhodesiense* have been met. The case-histories of these men are summarized below.

1. Volunteer 128 was not infected originally when fly GI. 2 ex sheep 302 fed on 26.2.42. On 28.3.42 two rats were each inoculated with 1 c.cm. of his undiluted blood. Both remained negative, and he was rebitten by fly GI. 2. After the rebite he had no reaction on his arm and no rise of temperature, and on the 27th day one rat was inoculated with 1 c.cm. of undiluted blood, and he was treated. The rat was infected, with an incubation-period of 27-28 days.

2. Volunteer 190 was not infected originally when fly IN. 11 ex sheep 327 fed on him on 10.4.43. On 8.5.43 five rats were inoculated, each with 1 c.cm. of undiluted blood. All remained negative, and he was rebitten by fly IN. 11. Five days after the rebite a nodule appeared on his arm, which developed into a typical reaction, puncture-positive on the 12th day. On the 14th and 15th days his temperature was 98.8° F. and 102.6° F., but no trypanosomes were found microscopically in his blood. By the 16th day his arm-reaction had disappeared and his temperature was normal, and remained normal until the 35th day, when four rats were inoculated and he was treated. The four rats were infected, with incubation-periods of 24-30, 24-30, 24-30 and 31-38 days.

3. Volunteer 229 was fed on by fly MO. 30 ex sheep 338 on 21.4.44. From the 3rd to the 9th days inclusive there was a soft raised swelling on his arm, but trypanosomes were not found on puncture. On the 8th and 9th days he complained of malaise, his temperature was normal, and

malarial parasites were found in his blood. Between the 16th and 19th days inclusive a soft diffuse swelling reappeared on his arm, but trypanosomes were not found either microscopically or by inoculation of rats, and the swelling was gone by the 20th day. On the 45th day two rats were inoculated, each with 1 c.cm. of undiluted blood, and he was treated on the 48th day. When the rats were examined 30 days later, they were found to be infected. At no time had this man had a temperature, and he felt perfectly well except for the two days' malaise.

4. Volunteer 231 was fed on by fly MO. 30 ex sheep 338 on 26.4.44. From the 9th to the 14th days a typical reaction was present on his arm; this was punctured daily, and trypanosomes were found on the 10th and 14th days only. By the 15th day the reaction had disappeared and did not return. From midday of the 10th to the morning of the 15th day his temperature was raised, reaching a peak of 104.6° F. on the evening of the 12th day. Trypanosomes were only found in his blood on the evenings of the 14th (1/200 fields) and 15th days (4/71), when his temperature was 104° F. and 97.6° F. respectively. As it was our custom to keep patients under observation until their blood proved positive in the morning, he was not treated at the time. At no subsequent date did he have a temperature. He felt perfectly well, and no trypanosomes were again seen microscopically in his blood. Laboratory animals were, however, inoculated periodically with his blood, and the following results were obtained:

15th day (morning),	2 rats, both negative after 69 days.
16th	" 2 " " " 68 "
17th	" 2 " " " 67 "
22nd	" 2 " " " 61 "
23rd	" 2 " one positive, incubation-period 41-47 days.
27th	" 2 " " " 35-43 "
33rd	" 2 " " " 31-37 "
40th	" 2 " both negative after 59 days.
52nd	" 2 " positive, incubation periods 10-12 days.
59th	" 2 " " " 15, 16 "
64th	" 2 monkeys inoculated with 2½ c.cm. blood each, both positive, incubation-period 7 days.

When the rats inoculated on the 52nd day, i.e., on 17.6.44, were found to be infected on the 64th day, i.e., on 29.6.44, the volunteer was immediately called to the laboratory and treated. The rats inoculated on the 23rd, 27th and 33rd days had been examined on 26.6.44 and 28.6.44 and were still not infected, and only became positive after those inoculated on the 52nd day.

It is interesting to note that, although this man's blood had visible trypanosomes on the evenings of the 14th and 15th days, yet rats inoculated on the mornings of the 15th and 16th days were not infected; it is also interesting to note the progressively shortening incubation-periods in the rats inoculated on the 23rd, 27th, 33rd and 52nd days of the disease.

These four atypical infections all occurred in men infected from the sheep line, when the trypanosome had become less virulent. A fifth case had a different history.

In 1945 volunteer 280 was one of six men out of 10 who were infected by fly PP. 33 from Thomson's gazelle 75. Clean laboratory-bred flies were fed on his thigh on the day when his blood was first found positive, and two infected flies, QE. 27 and QE. 16, were isolated. QE. 27, a moderately heavily infected fly, fed on a further 10 volunteers, and three of them were infected, with typical acute attacks of the disease. Rats inoculated from their arm-reactions and with their blood were all infected (Table X). The passage of the trypanosome through man had not increased its infectivity to man. QE. 16 was a very lightly infected fly, totals of 3, 3, 32, 7, 27, 59 metacyclic trypanosomes being counted on successive days, on each day a long series of slides being probed. After this, volunteer 334 was bitten. The next time the fly wanted to feed, a probe-test was done, and 24 metacyclic trypanosomes were counted. On subsequent consecutive hunger days the fly fed on volunteer 335; another probe-test gave five metacyclic trypanosomes; and, finally, volunteer 336 was bitten.

To test our theory that a minimum infective dose of metacyclic trypanosomes is required to infect man, the fly was to have been fed on a series of volunteers, but it died after having bitten only three men. The first of these, volunteer 334, was infected, and his history is as follows:

Volunteer 334 was fed on by fly QE. 16 on 9.5.45. From the 10th to the 18th days a very soft diffuse swelling, reaching a diameter of $\frac{1}{2}$ in., was present on his arm, the reaction being puncture-positive on the 10th, 11th, 13th, 15th and 16th days. On the 19th and 20th days the swelling had almost completely disappeared, leaving a suggestion of thickening of the skin. From the 21st to the 23rd days a firm lump appeared, which was negative for trypanosomes on daily puncture, reaching a size of about 1 in. \times $\frac{1}{2}$ in., and decreasing again between the 24th and 28th days until it was only a nodule about $\frac{1}{2}$ in. in diameter. On the 29th day a typical, firm, elastic reaction commenced, reaching a maximum size of 3 in. \times 2 in. on the 33rd day, with vesiculation of the skin on the surface. This reaction was puncture-positive on the 29th-32nd days inclusive. From the 34th to the 39th days the reaction grew softer and smaller, and the skin desquamated, but it was never smaller than 1 in. in diameter. From the 40th to the 45th days another reaction appeared, above the desquamated area of skin, reaching a maximum of 2 in. \times 1 in. on the 42nd day. It then receded, but was still about $\frac{1}{2}$ in. in diameter when the man was treated on the 45th day. This last reaction was not punctured. Rats were inoculated from the arm-reaction whenever trypanosomes were seen microscopically, and only five out of the 18 rats were infected (Table X).

On the evening of the 36th day his blood was positive for trypanosomes (3 in 66 fields), and was positive on the 37th-39th days, negative from the 40th to the 43rd day, and positive on the 44th and 45th days. During the whole of this period the patient never made any complaint of being ill, and the only occasions on which he had a raised temperature were on the 33rd day (98.6° F.) and the 38th day (99.0° F.). His temperature was 98.4° F. on the 45th day, when trypanosomes were present and he was treated.

The fact that volunteer 334 fell ill when a very lightly infected fly fed on him does not invalidate the hypothesis that the *average* man requires a minimum infective dose of over 300 metacyclic trypanosomes for infection. Volunteer 12 was infected by a dose of 170 metacyclic trypanosomes, and had a typical acute attack of the disease. The clinical history of volunteer 334 shows a trypanosome having great difficulty in establishing itself in man; and it is suggested that volunteer 334 had a comparatively low resistance to infection, but received a dose of trypanosomes somewhat higher than the minimal infecting one.

Volunteer 280 had been infected from the Thomson's gazelle line, which has been shown to have been of high virulence for animals. Fly QE. 27 derived its infection from man, and by definition this man must have been infected with *T. rhodesiense*. Yet it only infected three out of the 10 men on whom it fed. The failure to infect the other seven men cannot be ascribed to any accidental admixture of a strain of *T. brucei*, since the disease in the three infected men was typical of an acute infection by a virulent trypanosome. Fly QE. 16 derived its trypanosomes from the same human, and it is difficult to believe that the anomalous disease in volunteer 334 could be ascribed to any lack of virulence of the trypanosome.

The five men had to be treated comparatively early, as we dared not risk incurable complications; but they do show that under certain circumstances *T. rhodesiense* can produce an almost avirulent infection of man. The laboratory infection of volunteer 334, and to a lesser extent those of volunteers 229 and 231, appear to be similar to those infections found in the field and described as 'carrier' cases (Lamborn and Howat, 1936; Blair, 1939); and, if the analogy is accepted, it shows that there is no necessity to ascribe avirulent human infections found in a *rhodesiense* area as being due to *T. brucei* acclimatizing itself to man.

IX. DISCUSSION

A re-examination of Duke's records in the light of our work enables his anomalous results to be explained logically; but conclusions are arrived at radically different from his. Duke (1933*b*) had noted that, while strains Tinde II and Tinde III produced a heavy gland infection, the trypanosomes of strain Tinde I were always much less numerous in

the salivary glands, and he reached the conclusion that this sparse type of gland infection was a definite character of the strain. When, at a later date (Duke 1935*b*), he discussed the infectivity of strain Tinde I to man, he again remarked upon the lightness of the salivary gland infections, but went on to say: 'The readiness with which flies carrying strain Tinde I infect guinea-pigs, monkeys and antelope, shows that the failure with man cannot be ascribed solely to the inadequacy of the inoculum introduced when flies carrying this strain bite man.' Duke apparently assumed the infective dose of metacyclic trypanosomes for man to be of the same order as for a rat, a guinea-pig or an antelope, while in actual fact it is much higher. Even the subcutaneous injection into man of the whole of both glands of a fly infected with strain Tinde I (*loc. cit.*) was no guarantee that an infective dose of metacyclic trypanosomes had been inoculated.

He also referred (Duke 1935*a*) to a volunteer who had previously resisted a thorough exposure to strain Tinde I, and who was subsequently bitten and infected by two flies carrying Tinde III, and he remarked that this was the only instance yet recorded where two strains of *T. rhodesiense*, both of which had resided for some time in antelope and had been removed for months from contact with man, behaved differently in the same human subject. When Duke's own account of the difference of the salivary gland infections produced by the two strains Tinde I and Tinde III is borne in mind, it is understandable that, although the bite of one fly carrying strain Tinde I might not be infective, the bites of two flies carrying strain Tinde III might well inoculate an infective dose of metacyclic trypanosomes.

Referring to volunteers A and B infected with strain Tinde III, Duke (1935*b*) stated that both these volunteers escaped infection when bitten by flies carrying trypanosomes derived directly from bushbuck I, but both these men and volunteer C all became infected when bitten by flies infected from the two monkeys 1154 and 1157, which in their turn had been infected from the bushbuck directly by the syringe, and he remarked that 'the stay in the monkey may have prepared the trypanosome against man's tissues.'

It is quite impossible for anyone to say from which host volunteers A and B were infected. Duke maintained that to attribute the infection of A and B to the flies of the bushbuck test involved the assumption of an incubation-period of 28 days for A and 20 days for B, and he considered this both unnecessary and unjustifiable; but we have seen that one of our volunteers, volunteer 138, who was infected from Thomson's gazelle ex sheep, had a known incubation-period of 20 days, while volunteer 128a, infected from sheep, had no signs or symptoms on the 27th day, and yet his blood was infective to a rat.

Volunteers A and B may have been infected by flies from monkeys 1157 and 1154 respectively, as Duke maintained; but, if this is so, it probably means that the flies infected from the monkeys merely inoculated a larger dose of metacyclic trypanosomes than the flies infected from the bushbuck (compare our results with the sheep line on transference to monkeys in 1940). There is thus no need at all to postulate any preparation of the trypanosome against man's tissues by its residence in monkeys.

Two years after he had stated 'that the ability [of strain Tinde I] to infect man has been lost is quite certain,' Duke (1937) reported that it had infected two volunteers, who had each been inoculated with 1 c.cm. of citrated monkey blood. To explain this result, he postulated that either (*a*) the trypanosome during its residence in bushbuck II had reacquired the power of infecting man, or (*b*) the forms in the monkey's blood were infective to man, whereas the metacyclic forms tested in the earlier investigation were not. Neither of these postulates is necessary.

Duke (1935*b*) reported that 12 out of 17 volunteers infected showed no local reaction at the site of the bite—in contradistinction to our results, where only 17 out of 206 infected men had no reaction. If the conclusion is correct that the absence of a reaction at the site of the infective bite is due to the man's receiving a bare minimal infecting dose of metacyclic trypanosomes, then Duke's results point to the fact that most of the infected flies he used were inoculating low doses.

Duke thought that when infected flies failed to infect man this was an indication that the trypanosomes had lost their infective quality. But fly QE. 27, which had derived its infection from man, volunteer 280, only infected three out of the 10 men bitten. Our work shows, moreover, that, provided a large enough number of men are bitten, the infectivity of a line to man can be demonstrated even when infectivity is low.

Duke's results do not prove that *T. rhodesiense* can revert, or was reverting, to *T. brucei*. They can be explained by the fact that man requires a comparatively large dose of metacyclic trypanosomes in order to be infected, that various host animals influence transmissibility and dosage appreciably, and that whether a fly inoculates an infective dose or not on biting man depends in large measure on the host animal from which it has acquired its infection.

In view of our failure to infect volunteers 83 and 98 by the inoculation of 102,000 trypanosomes from a rat, and the failure to infect volunteer 69 by the inoculation of 1,100 metacyclic trypanosomes (although he had been infected by the inoculation of 102,000 blood trypanosomes), the history of volunteer W (Duke 1935*a*, 1935*b*) is not outstanding, except to show that his resistance to infection was probably very high.

The transmissibility of a strain can be influenced by the temperature at which the flies are maintained in the laboratory, and, if laboratory-temperatures are low, the flies may have to be incubated in order to obtain optimum transmissions. Increased transmission-rates can also be obtained by incubating the pupae. By cooling them it may be found that the flies emerging give low transmissibility. The variation in the temperature to which tsetse pupae are exposed may be one of the causes of the difference in the percentage of wild flies found infected in the cool and the hot seasons. We consider that this influence of temperature, both on the pupae which are collected in the field and on the flies during maintenance in the laboratory, has not received the attention which it undoubtedly deserves in reports purporting to show that strains are losing their transmissibility (e.g., 'gut positive, salivary glands negative').

Lester (1933), on examining a number of Nigerian strains of *T. gambiense*, came to the conclusion that strains occurred possessing all the characteristics ascribed to *T. rhodesiense*, and that there was a series with characteristics intermediate between *T. rhodesiense* and *T. gambiense*. He concluded: 'The writer holds the view that *T. rhodesiense* is only a virulent type of *T. gambiense*, and that this more virulent type usually arises from the normal human trypanosome through idiosyncrasies in the resistance of the human host.' The characteristics of the Tinde strain of *T. rhodesiense* maintained in sheep shows that its virulence for sheep and rats has decreased, the incubation-period in men infected has increased, the clinical disease produced in man is milder, the trypanosome from man infected less than half the rats inoculated, and the disease in those rats which were infected had a prolonged incubation-period, was chronic and relapsing in type, while relatively few posterior-nuclear forms were present. By the accepted criteria for differentiating *T. rhodesiense* and *T. gambiense*, it would appear as if this line of a known *T. rhodesiense*,

maintained in sheep, were approaching a *gambiense* type; but it has taken 10½ years' maintenance in this single species of host to produce this change. The maintenance of the same strain in antelope for nine years and in monkeys for five years has produced no alteration in its characters.

It is fully realized that our work has been based on the examination of a single strain of *T. rhodesiense*. The differing alterations in virulence produced in the sheep, monkey and Thomson's gazelle lines are probably due to gene mutations; and, should other strains of *T. rhodesiense* be examined for a comparable period of time, it is likely that different results would be obtained owing to other chance mutations; but this strain has been passaged for nearly 11 years solely by cyclically infected *G. morsitans*, and intensive investigation of it has led to certain deductions being drawn. Prolonged maintenance of strains by syringe passage is unnatural and tends to the production of unnatural characters. We therefore consider that to obtain valid laboratory results, capable of interpreting field-conditions accurately, all future work on trypanosomiasis should be done with strains transmitted by the insect vector.

In an important paper, Hoare (1943) has stated the case for the recognition in protozoa of 'biological races,' i.e., organisms which are indistinguishable morphologically but which show specific serological differences, differences in their localization in definite organs or tissues, in their host-restrictions and in their vectors. In many of these biological races the specific differences depend upon differing chemical structures of the antigenic components, and are apparently part and parcel of their genetic make-up, being hereditarily 'fixed.' He therefore suggests the desirability of assigning biological races to an independent systematic position, with a distinct name.

We are not prepared to comment on the relationship of *T. rhodesiense* and *T. gambiense*. Our results, however, strongly support the view that *T. rhodesiense* and *T. brucei* must be considered as being distinct, and not convertible into one another. Whether they are called biological races or species is a matter for the systematists.

X. SUMMARY

1. A strain of *Trypanosoma rhodesiense*, passaged by cyclically infected *Glossina morsitans* through various animals for 10½ years, was still infective to man.
2. Maintenance in a single species of host resulted in a drop of infectivity to man taking place, but this recovered after further maintenance in the same host species.
3. Men, who failed to be infected when fed upon by an infected fly, were frequently infected when rebitten by the same fly after an interval of some weeks.
4. Metacyclic trypanosomes were counted in drops of fluid and definite numbers inoculated into human volunteers; it was found that the average man required a dose of 300-450 metacyclic trypanosomes in order to be infected, but that there were men whose infective dose was higher or lower than this.
5. Some case-histories are recorded showing the high resistance to infection possessed by some men.
6. A method is reported by which infected flies were induced to probe on to albumen-smear slides. This technique allowed the infection in the living fly to be studied, and gave data on the probable number of metacyclic trypanosomes extruded when the fly fed.
7. It was found that the number of metacyclic trypanosomes ejected was low, and more constant, in the first half of the fly's infected life, and high, and more variable, in the second half.

8. The degree of infection of the salivary glands of *G. morsitans*, and hence the ability of the flies to inoculate an infective dose of metacyclic trypanosomes, might vary from fly to fly in the same isolation, and from one isolation to another.

9. The transmissibility of the strain (i.e., its ability to invade the salivary glands of the fly) and its infectivity to man were correlated significantly.

10. Transmissibility was influenced by the host from which the flies were infected, and also by the temperature conditions to which the flies were exposed.

11. Flies emerging from pupae which had been incubated at approximately 30° C. gave significantly higher transmission-rates than flies from pupae left under normal laboratory conditions. The trypanosome-cycle in flies from incubated pupae was also significantly shorter than in flies from normal pupae; and a significantly greater number of flies from incubated pupae survived to be isolated than from normal pupae.

12. The virulence of the sheep line had decreased between 1934 and 1945, and yet its infectivity to man was still high. The virulence of the Thomson's gazelle and monkey lines had increased between 1940 and 1945, and the infectivity to man had fluctuated over the same period.

13. A number of atypical infections of man with *T. rhodesiense* are recorded, and are compared with the 'carrier' cases of trypanosomiasis reported in the literature. It is concluded that there is no necessity to assume that these 'carrier' cases are examples of *T. brucei* becoming acclimatized to man.

14. The results of the inoculation of rats with positive blood from the arm-reactions and the blood of men infected from the various lines are recorded. It is suggested that *T. rhodesiense* maintained in sheep is assuming a *gambiense* character. This character has only become apparent after 10½ years' maintenance by cyclical transmission in a single species of host.

15. Flies infected from man only infected a small proportion of the men on whom they fed. The failure of man to be infected by a fly carrying a known *T. rhodesiense* does not necessarily mean any alteration in the infective quality of the trypanosome.

16. Some previously published work on the relationship of *T. rhodesiense* and *T. brucei* is reviewed in the light of our results, and the conclusion is drawn that *T. rhodesiense* and *T. brucei* must be considered as distinct, and not convertible into one another.

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APPENDIX A
The length of life of infected sheep and monkeys, in days

1934	1935	1936	1937	1938	1939	1940		1941		1942		1943		1944		1945	
						Sheep	Monkeys	Sheep	Monkeys	Sheep	Monkeys	Sheep	Monkeys	Sheep	Monkeys	Sheep	Monkeys
69	152	104	83	245	151	294	338	114	183	346	86	242	171	335	93		61
91	110	99	177	131	139	82	239	169	267	163	230	243	151	178	42		43
214	115	176	141	156	379	49		399	185	476	122	337	115	258	67		35
123	91	93	106	194	159	284		232	69	285	112	225	76	395	53		111
	77	40	48	239	85	343		264	76	324	75	132	63	405	116		31
	82	97	58	232	386	136		228	141	388	174	259	96	397	59		33
		197	54	51	311	150		253	90	349	115	386	141	351	81		14
		166	77	211	135	356		334	109	410	114	351	128		100		80
		201	254	176	260	298		343		227	67	229	221		84		13
		223	109	137		337		38		376	168		207		33		58
		184	188	301		129		239		352	196		156		157		
		127	304	205		347		194		379	288		165		40		
		49	322			282		177		135	120		124		72		
		151	275			260		70		77	139		165		64		
		66	207					258		321	144		78		81		
		171	146							311	36		162		38		
		47								220	168		110		50		
										311	229		84		71		
										297	119		50		86		
										228			128		68		
										271			167		57		
													144		51		
													56				
													183				
													152				
													160				
													80				
													161				

All sheep still alive

The length of life of a sheep was the number of days between the time when a box of flies was transferred to it from the previous infected host and its death.

The length of life of a monkey was the number of days from the time when its blood was first positive on microscopical examination until its death.

APPENDIX B

The length of life of rats infected by the bite of *G. moritans*, themselves infected from the sheep, monkey and Thomson's gazelle lines

No. of rats dying in the following period of days	Rats infected from sheep line					Rats infected from monkey line					Rats infected from Thomson's gazelle line							
	1940	1941	1942	1943	1944	1945	1940	1941	1942	1943	1944	1945	1940	1941	1942	1943	1944	1945
1- 5 days	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6-10 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11-15 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16-20 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21-25 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
26-30 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31-35 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
36-40 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
41-45 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
46-50 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
51-55 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
56-60 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
61-65 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
66-70 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
71-75 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
76-80 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
81-85 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
86-90 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
91-95 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
96-100 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
101-105 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
106-110 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
111-115 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
116-120 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
121-125 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
126-130 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
131-135 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
136-140 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
141-145 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
146-150 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
151-155 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
156-160 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
161-165 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
166-170 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
171-175 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
176-180 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
235 "	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

APPENDIX C

The number of days taken by the trypanosomes of the sheep, monkey and Thomson's gazelle lines to complete the cycle in *G. morsitans*

Length of cycle	No. of transmissions from each line		
	Sheep	Monkey	Thomson's gazelle
18 days			1
19 "		1	2
20 "		5	-
21 "	1	3	2
22 "	2	4	2
23 "	4	5	7
24 "	-	5	6
25 "	1	5	4
26 "	5	7	4
27 "	2	5	6
28 "	3	4	3
29 "	1	3	5
30 "	4	1	5
31 "	-	3	4
32 "	1	1	1
33 "	4	-	-
34 "	-	1	1
35 "	1	1	1
36 "	4	2	2
37 "	-	1	
38 "	-	-	
39 "	4	1	
40 "	-	-	
41 "	1	1	
42 "	-	-	
43 "	-	1	
44 "	-	-	
45 "	1	-	
46 "	-	-	
47 "	-	1	
48 "	1	1	

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EXPERIMENTS WITH LEECH REPELLENTS

BY

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The experiments with leech repellents recorded below were made in order to determine the most effective means of combating land leeches, which were causing considerable casualties to troops campaigning in south-east Asia.

MATERIALS AND METHODS

The materials tested were dimethylphthalate (DMP), dibutylphthalate (DBP), Rutgers 612 (2-ethylhexanediol-1,3) and 5 per cent. dichlordiphenyltrichlorethane (DDT) in kerosene.

Haemadipsa sylvestris, the largest and most robust of the Indian land leeches (Moore, 1927), was the species used for the tests. These animals are very common amongst damp vegetation in Assam. They progress by a looping movement, with only their head- and caudal suckers in contact with the ground, so that they can cross any distance rather shorter than their stretched length without touching the intervening surface. The fully stretched length of the specimens used varied between $1\frac{1}{2}$ inches and 3 inches, and most of them could stretch more than 2 inches.

The leeches were not sufficiently abundant for field-tests, so a laboratory technique was devised. Sets of four strips of khaki drill, each strip 12 inches long and of standard width, were impregnated with the substance to be tested, and were then placed flat on damp bed-sheets, so that they formed a square box. The leeches were confined, one at a time, within these boxes, and the efficiency of the repellent was judged by the proportion of successful attempts to cross the strips. The experiments were conducted in the open, and the leeches made every possible attempt to get away from the unfavourable environment of the white sheet, so that their incentive to cross the strips to safety was at least as great as their natural urge to feed.

Even impregnation of the strips with minute quantities of repellent presented difficulties. Strips of zinc were cut, of the same size as the strip to be tested, and the dose was applied evenly to their smooth non-absorbent surface. The strips of drill were damped, in order to reduce their speed of absorption, placed on the treated zinc strips, firmly pressed down, and then finally rubbed over them. They thereby acquired a fairly even dose. Larger doses were applied in instalments, which also aided even impregnation.

When moving on a suitable surface a leech normally fixed its head-sucker wherever it first touched, but when confronted with an impregnated surface the sucker was withdrawn and touched another spot, and this process continued until an untreated spot was reached; the number of touches made before a suitable spot was found was largely dependent on the angle at which the leech approached the strip—sometimes it moved its head-sucker back into the untreated square, but sometimes the impregnated strip was touched many times before the sucker found a suitable unimpregnated resting-place. In each experiment a new attempt was considered to be made when the leech removed its caudal sucker and affixed this to another spot, and both the number of attempts to cross the strip and the total number of touches made by the head-sucker during these attempts

were recorded. In the tabulated results the record '2 in 20 (87)' would mean that the leech made 20 attempts to cross, in the course of which its head-sucker touched the impregnated strip 87 times, and only two attempts were successful.

REQUIRED WIDTH OF THE PROTECTIVE STRIP

Four sets of 12-inch strips, of widths $\frac{1}{2}$ inch, $\frac{3}{4}$ inch, 1 inch and $1\frac{1}{2}$ inches, were impregnated with 0.5 c.cm. DMP per strip, a dose which was completely repellent when touched, and tested.

Five leeches, whose average maximum stretched length was $2\frac{1}{2}$ inches, each made 10 attempts to cross each set of strips. The strips were tested dry, and were then wetted and retested, with the results shown in Table I. In every case where the leech crossed the strip it stretched across it without touching it.

TABLE I

Comparison of strips of different widths impregnated with DMP (0.5 c.cm. per 12-inch strip)

Width of strip	$\frac{1}{2}$ inch	$\frac{3}{4}$ inch	1 inch	$1\frac{1}{2}$ inches
No. of attempts	50	50	50	50
Dry strips : times crossed	26	21	7	0
Wet " : " "	2	Not done	0	0

Conclusion. The minimum barrier-width for complete repellency, using dry materials, was $1\frac{1}{2}$ inches. When the cloth and surroundings were damp, a narrower width sufficed, because DMP spread as a surface-film over the wetted areas contiguous to the strips, and thereby enlarged their effective width.

REPELLENT EFFECT OF DMP

(a) *Minimum Effective Dose*

Sets of four strips, each strip 12 inches by $1\frac{1}{2}$ inches, were impregnated with quantities of DMP varying between 0.0075 c.cm. and 2.0 c.cm. per strip, and each set of strips was tested three times soon after treatment, using a different leech each time. The results are shown in Table II.

Conclusion. These experiments show that the minimum completely effective dose of DMP under these experimental conditions was at the rate of 0.5 c.cm. per 12-inch by $1\frac{1}{2}$ -inch strip, but that much smaller doses give a very high degree of repellency. With a dose of only 0.0075 c.cm., 82 per cent. of all attempts to cross failed, and 97 per cent. of touches by the head-sucker led to withdrawal. The very high proportion of repellency for this minute dose indicates that the DMP was effective at this concentration, and that the successful crossing of the strip was due to its imperfect impregnation by so small a quantity.

(b) *Duration of DMP Repellency in Shade*

The impregnated strips were kept in the shade, and each set of strips was tested daily with two leeches until it became ineffective. Each leech was required to make 20

TABLE II
Showing the effectiveness of varying quantities of DMP

Dose	Separate experiments	Total crossings and attempts	Percentage of attempts repelled	Percentage of touches repelled
0.0075 c.cm. (= 0.06 c.cm./sq. ft.)	a. 2 in 20 (61) b. 3 „ 20 (109) c. 6 „ 20 (167)	11 in 60 (337)*	82	97
0.03 c.cm. (= 0.24 c.cm./sq. ft.)	a. 0 in 20 (76) b. 2 „ 17 (92) c. 4 „ 20 (134)	6 in 57 (302)	89	98
0.125 c.cm. (= 1 c.cm./sq. ft.)	a. 1 in 20 (53) b. 2 „ 20 (116) c. 3 „ 20 (112)	6 in 60 (281)	90	98
0.5 c.cm. (= 4 c.cm./sq. ft.)	a. 0 in 10 (66) b. 0 „ 10 (59)	0 in 20 (125)	100	100
2.0 c.cm. (= 16 c.cm./sq. ft.)	a. 0 in 10 (14) b. 0 „ 10 (15)	0 in 20 (29)	100	100

* 11 in 60 (337) means that the leech made 60 attempts to cross, of which 11 were successful, and that the head-sucker touched the impregnated strip 337 times.

attempts on each occasion. The percentage of unsuccessful attempts to cross is shown in Table III.

The weather-conditions during these and the subsequent experiments were :

Night temperature, minimum 70°F.
Day „ „ 82°F.
„ „ maximum 93°F.
„ Relative humidity, C. 90 per cent.

TABLE III
Showing the duration of repellency of DMP in shade

Dose	Percentage of repelled attempts									
	Same day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
0.0075 c.cm.	82	Nil								
0.03 „	89	22	Nil							
0.125 „	90	90	45	17	Nil					
0.5 „	100	100	100	100	100	100	Nil			
2.0 „	100	100	100	100	100	100	100	100	100	100
	11th day	12th day	13th day	14th day	15th day	16th day	17th day	18th day	19th day	
2.0 „	100	95	90	100	100	100	100	12½	Nil	

(c) Duration of DMP Repellency in the Sun

The previous series of experiments were repeated with the sets of strips fully exposed to sunlight throughout the day. Results are shown in Table IV.

TABLE IV
Showing the duration of repellency of DMP in the sun

Dose	Percentages of repelled attempts									
	Same day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
0.0075 c.cm.	82	Nil								
0.03 "	89	52	Nil							
0.125 "	90	100	72	Nil						
0.5 "	100	100	100	100	10	Nil				
2.0 "	100	100	100	100	100	100	100	100	65	Nil

Comparison of Table III and Table IV shows that, as would be expected, exposure to sunlight markedly reduced the duration of the effectiveness of DMP. Unfortunately, the daily strength and duration of sunlight were very variable, and no apparatus was available to enable quantitative estimates to be made. The average quantity of sunshine during these experiments was about five hours per day, and under these conditions the sunlight never reduced the duration of repellency by as much as 50 per cent.

(d) *Effect of Washing on DMP Repellency*

To test the effect of laundering on DMP, a standard method of washing was used : at each wash each strip was wetted, rubbed three times with moist soap, then 10 times rolled in the hands and dipped into water, then shaken 10 times in clean water, then removed and squeezed dry, and twice redipped in water and resqueezed. Each strip was tested by two leeches after each washing. The results are shown in Table V.

TABLE V
Showing the effect of washing on DMP repellency

Dose	Percentage of repelled attempts			
	After 1st wash	After 2nd wash	After 3rd wash	After 4th wash
0.03 c.cm. ...	Nil			
0.125 " ...	100	Nil		
0.5 " ...	100	100	73	Nil
2.0 " ...	100	100	100	Nil

(e) *Effect of Running Water on DMP Repellency*

Troops may be exposed to water for considerable periods, either in heavy monsoon rains or in swamps. Treated strips were therefore placed in very slowly running water and tested by two leeches at hourly intervals thereafter. The results obtained are shown in Table VI.

TABLE VI
Showing the effect of slowly running water on DMP

Dose	Percentage of repelled attempts			
	After 1 hour	After 2 hours	After 3 hours	After 4 hours
0.125 c.cm. ...	Nil			
0.5 " ...	100	Nil		
2.0 " ...	100	100	60	Nil

COMPARISON OF DMP WITH DBP, RUTGERS 612 AND DDT

Comparison of DMP with DBP shows that the latter is almost useless as a leech repellent. Testing of strips impregnated with 2.0 c.cm. of DBP showed that they were only 62 per cent. effective immediately after treatment (two leeches made 15 crossings in 40 attempts) and that they were completely ineffective after one day in the sun, after one wash, and after one hour of exposure to running water. The relative lethal effects of the two liquids were also investigated, and whereas leeches placed on a drop containing 0.025 c.cm. of DMP were immobilized in $1\frac{1}{2}$ –3 minutes, and completely dead in 5–8 minutes, twice this dose of DBP took 18–45 minutes for immobilization and 24–53 minutes to kill the leeches completely.

Comparison of DMP with Rutgers 612 showed that the latter was at least as effective as DMP at the time of application (100 per cent. repellency with a dose of 0.125 c.cm.) but that its effect wore off much more rapidly—strips treated with 0.125 c.cm. of Rutgers 612 were only 5 per cent. effective after being kept for 24 hours in the shade, and strips treated with 2.0 c.cm. were completely ineffective after one washing or one hour in slowly running water.

Impregnation of strips with 5 per cent. DDT in kerosene was ineffective: 0.5 c.cm. per strip had no repellent effect, and impregnation with 2 c.cm. per strip was only 35 per cent. effective, although the leeches did behave abnormally after crossing this concentration.

DISCUSSION

These results prove that DMP is a very effective land-leech repellent, but they show that the duration of its efficiency varies considerably with prevailing conditions, such as quantity of sunlight, effect of heavy rain or of wading through swamps, and laundering of the impregnated surface. Exact quantitative estimates of the relative importance of these different factors in reducing the repellency of the DMP cannot be made, but the results from the impregnation of strips with 2.0 c.cm. DMP may be taken as a guide; four hours in very slowly running water was equivalent to four launderings with soap and water, or to nine days' exposure to moderate sunlight, or to 18 days' exposure in the shade. Results showed that immediately after application very minute quantities of DMP were effective repellents, and the minimum dosage was therefore the smallest quantity which could be applied so that it covered the surface. This dose was 0.5 c.cm. per 12-inch by $1\frac{1}{2}$ -inch strip, but 0.0075 c.cm. was 82 per cent. effective on similar strips. Larger doses could be applied more evenly, and once applied they remained evenly distributed during subsequent exposure, so that heavily treated strips remained fully effective for a long time and then suddenly became completely ineffective.

Other workers have shown in unpublished reports that applications of DMP to clothing provides effective protection against mosquitoes and trombiculid mites for long periods, and therefore in some cases a general application of DMP would serve a threefold purpose. Probably the effects of exposure to sunlight, laundering and water are similar in principle in all cases, but the threshold dose for control may be expected to vary.

Several tests, not here recorded, showed that DMP was very effective against aquatic leeches also.

SUGGESTED ANTI-LEECH MEASURES

When walking through short grasses full protection has been obtained by the application of a thin smear of DMP in a $1\frac{1}{2}$ -inch band round the neck of shoes or boots and to their

tongues and lace-holes. While conducting larvicide experiments in a stream in Ceylon a party of three people were all bitten by land leeches in a few minutes. Next day the same three people returned, and worked in the infested locality for several hours. Two persons, wearing shoes, applied the DMP as described above, and were completely protected. The leeches, which were of the rather small gregarious species *Haemadipsa zeylanica*, perambulated round and round the shoes, making unsuccessful efforts to cross the treated area; they were so abundant that in one spot the average number attaching was 25 per shoe. The third member of the party was wearing Army boots; he applied the band of DMP but omitted to treat the lace-holes. He received six bad bites on his feet from leeches which had entered his boots through the lace-holes.

Where individuals have to move through or bivouac in tall leech-infested vegetation, treatment must be a matter of common sense and must be dependent upon circumstances, but it should always be remembered that, wherever possible, the DMP should be applied to the parts of clothing which are least exposed to the elements.

As the duration of protection is largely dependent upon external conditions, no definite suggestions for renewal can usefully be made; but fortunately this is not essential, because leeches are not disease-carriers, and retreatment can be carried out as soon as it is found that previous treatment has ceased to be completely effective.

SUMMARY

Dimethylphthalate was proved to be a very effective and durable land-leech repellent. A dose at the rate of 4 c.cm. per square foot, applied to cloth in a band $1\frac{1}{2}$ inches wide, was completely repellent, under favourable circumstances, for six days. A dose at the rate of 0.06 c.cm. per square foot was more than 60 per cent. effective immediately after similar application.

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FAILURE TO CURE NATURAL CANINE VISCERAL LEISHMANIASIS

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During the last seven years we have had the opportunity of treating naturally acquired canine visceral leishmaniasis and of collecting information on the subject from practising veterinarians in Palestine. We have been struck by the failure of treatment both with antimony compounds and with aromatic diamidines. It is not difficult to produce clinical improvement with either series of drugs, particularly if chemotherapy is associated with a liberal diet of fresh meat; but it does not seem to us feasible, with the methods at our disposal, to eradicate the infection from the lymphatic glands, the skin and the corneoscleral junction. Even in cases in which no parasites were found in spleen smears after intensive treatment it was possible to obtain a culture from splenic juice, and parasites could be demonstrated in smears of lymphatic glands and in sections of the normal unbroken skin. The presence of a residual, though diminished, skin-infection after intensive treatment is an obvious proof of failure, in spite of clinical improvement, for under natural conditions the transmitting sandflies infect themselves from the skin-juices of infected dogs. Throughout the whole Mediterranean basin the unbroken skin of naturally infected dogs is the reservoir—and, as far as present knowledge goes, the only reservoir—from which sandflies of the *major* group infect themselves with *L. infantum* and transmit the disease to dogs and man.

The following brief notes from our protocols on the treatment of naturally infected animals in Palestine are instructive.

1. MALE BOXER. Aet. 3 years. Weight 24 kilo.

On February 2nd, 1939, visceral leishmaniasis was diagnosed, and the animal was given a course of 50 injections of neostibosan, each of 0.3 gm., three times weekly (i.e., a more intense course of treatment than is usually given to man). After each 20 injections there was an interval of two weeks. Marked clinical improvement was observed after the first 20 injections.

The animal deteriorated shortly after cessation of treatment and was brought to the laboratory.

Condition on Admission. Conjunctiva injected. Oedema of face and legs. Left popliteal gland enlarged. Skin showed marked depilation. Numerous small reddish nodules on the skin; on section these were found to consist of a mass of heavily infected reticulum. Skin also showed ulceration in parts. Skin smears showed a heavy infection, and parasites were found not only in macrophages but also in polymorphs.

2.10.39. 72 mgm. 4 : 4'-diamidino stilbene in 25 c.cm. distilled water was slowly injected intravenously. After the injection the pulse was slow, breathing was deep, and the animal was unconscious for 20 minutes.

4.10.39. Injection repeated. Reaction much slighter than after the first injection.

6.10.39. Animal depressed. Abdomen tender.

7.10.39. Diarrhoea with blood and mucus.

8.10.39. Slight improvement.

9.10.39. Cutaneous ulcers showed signs of healing.

16.10.39. 48 mgm. 4 : 4'-diamidino stilbene injected intravenously.

17.10.39. Smear from skin of ear showed numerous Leishman-Donovan bodies.

18.10-13.11.39. Received a total of 14 injections, each of 75 mgm. 4 : 4'-diamidino stilbene.

20.10.39. Received 0.05 gm. arecoline and passed a large number of cestodes (*Dipylidium caninum*).

22.10.39. Skin infection still intense.

29.10.39. Smears from cutaneous nodules showed numerous parasites. During these 14 injections the animal showed marked anorexia. Received insulin and intravenous glucose daily.

1.12.39. Marked swelling in maxillary and parotid region.

13.12.39. Smears from left popliteal gland showed numerous Leishman-Donovan bodies.

Between December 14th and 31st the animal received another 12 intravenous injections, each of 60 mgm. 4 : 4'-diamidino stilbene.

19.12.39. Number of Leishman-Donovan bodies in smear from left popliteal gland reduced. Smears from cutaneous nodules negative.

20.12.39. Histological examination of skin removed at biopsy showed a considerable, though reduced, number of Leishman-Donovan bodies.

Throughout the whole course of treatment the globulin-albumin ratio was high, but it diminished slightly towards the end, e.g., on November 17th, 1939, globulin 8.9, albumin 0.8 gm. ; on January 2nd, 1940, globulin 8.45, albumin 1.7 gm. per 100 c.cm. serum.

The formol-gel reaction was always instantaneous. Blood calcium varied from 7 to 9 mgm. per 100 c.cm.

5.1.40. Animal found dead.

Post-Mortem Examination. There was an extensive haemorrhage from the jugular veins. This haemorrhage was probably related to previous trauma, for numerous small shot were found in the muscles of the neck. Smears from spleen and liver were negative, but cultures from both organs were positive.

Although the number of parasites in the skin was further reduced they were easily demonstrated in sections. The skin-infection was of the type that in the light of previous experience could be estimated to produce an infection-rate of over 30 per cent. in sandflies (*P. perniciosus*). The lymphatic glands showed numerous parasites, and, as is often the case in infected dogs, some macrophages, both infected and non-infected, showed an indiscriminate phagocytosis engulfing red cells, leucocytes, and even other infected macrophages. Numerous infected macrophages were found in the corneo-scleral junction. No parasites were found in histological preparation of the liver and bone-marrow or in spleen smears. The liver showed distinct fatty degeneration.

Remarks. In spite of intensive treatment (including a total of 2.0 gm. 4 : 4'-diamidino stilbene) the infection was not eradicated and parasites could easily be demonstrated microscopically in the skin, lymphatic glands and corneo-scleral junction, and cultures from liver and spleen were positive.

2. MALE DOBERMAN. Weight 29 kilo.

Emaciated, in spite of a good appetite. Seborrhoea ; small cutaneous ulcers near both eyes ; parasites found in smear from left popliteal gland ; albuminuria.

Between October 29th and December 7th, 1939, the animal received 15 intravenous injections of 4 : 4'-diamidino stilbene in distilled water, and between December 19th, 1939, and January 11th, 1940, another 11 intravenous injections. The dosage varied from 35 to 100 mgm. per injection ; altogether a total of 1.8 gm. of the drug were administered.

5.11.39. After the third injection there was loss of appetite.

6.11.39. Unsteady gait.

From 7.11.39. Calcium gluconate given daily intravenously.

26.11.39. After nine injections there was no appreciable change in number of parasites in smear from left popliteal gland.

7-10.12.39. Anorexia and subsequent improvement. Treatment stopped and resumed on December 19th.

17.12.39. After 15 injections there was no appreciable diminution of parasites in smears from popliteal gland.

After the last 11 injections anorexia again reappeared and treatment was stopped. There was no appreciable diminution in parasites in the popliteal gland, and the animal was destroyed on February 2nd, 1940.

Post-Mortem Examination. The pertinent findings on post-mortem were as follows. Leishman-Donovan bodies were found in the lymphatic glands, spleen and bone-marrow. Histological examination showed that the skin, even in parts which were superficially normal, was infected. The infection of the tarsi was particularly heavy. The corneo-scleral junction was infected. It is interesting to note that numerous infected macrophages were found lying among fat-cells in the omentum. No parasites

were found in the liver, which showed a very marked fatty degeneration. Since marked fatty degeneration is not a feature of canine visceral leishmaniasis, it was probably caused by the drug.

Throughout the whole course of treatment the formol-gel reaction was almost instantaneous, and the globulin-albumin ratio was high and even increased towards the termination of treatment (on December 18th, 1939, globulin 4.7, albumin 2.4 gm.; on January 7th, 1940, globulin 6.2, albumin 2.2 gm. per 100 c.cm. serum).

3. MALE BOXER. Weight 20 kilo. General condition good. Lymphatic glands enlarged.

8.2.40. Smear from popliteal gland showed numerous Leishman-Donovan bodies (50 per 100 nuclei).

31.3.40. Animal laparotomized and spleen found enlarged and hard. Leishman-Donovan bodies 25 per 100 nuclei.

Between May 16th, 1940, and January 21st, 1941, the animal received 50 injections of 4:4'-diamidino stilbene, each of 1 mgm. per kilo. body weight. There were intervals in treatment owing to anorexia. Up to July 25th the injections were carried out every alternate day. By July 20th there had been a fall in the number of parasites to 5 per 100 nuclei in smears from the popliteal gland.

The first injection was followed by vomiting and a transient paraplegia. After subsequent injections there was no reaction. Injections were stopped because of anorexia.

12.8.40. The infection in the popliteal gland was 3 per 100 nuclei (after 24 injections).

19.8.40. Oedema extending over the whole back and sides.

29.8.40. Infection in popliteal gland 3 per 100 nuclei. Injections resumed. The animal had lost its tolerance for the drug, but the reaction was smaller than that following the first injection.

27.10.40. Infection in popliteal gland. Leishman-Donovan bodies 3 per 100 nuclei. The injections were again stopped because of anorexia.

18.11.40. Infection in popliteal gland 8 per 100 nuclei. Ulcers appeared on the eye-lids.

2.12.40. Injections resumed and given daily up to December 6th. All the injections were followed by tetany and shock (blood calcium 8).

From December 8th to 18th the animal received daily intravenous calcium gluconate 5 c.cm. (10 per cent.). The calcium injections were accompanied by a noticeable clinical improvement.

31.12.40. Smear of popliteal gland showed 25 Leishman-Donovan bodies per 100 nuclei.

21.1.41. Treatment resumed, 2 mgm. per kilo. body weight (50 mgm.) in 10 c.cm. 10 per cent. calcium gluconate and 5 c.cm. distilled water.

This injection was followed by unconsciousness and cessation of respiration and muscular tremors. The animal recovered after 15 minutes' artificial respiration.

22-24.1.41. Injection, repeated daily, was not followed by the slightest reaction.

27.1.41. Animal died.

Post-Mortem Examination. Number of parasites in popliteal gland 30 per 100 nuclei. Haemorrhages in large and small intestines. Liver enlarged threefold. Spleen twice the normal. Pneumonia. Kidneys showed long-standing chronic interstitial nephritis.

4. FEMALE BOXER. Aet. 5 years. Weight 23 kilo. Pregnant.

The animal came to the laboratory on January 8th, 1941, and treatment commenced three months after the first signs of illness. The number of parasites in popliteal smears were more than 100 Leishman-Donovan bodies per 100 nuclei.

21.1.41. Received an injection of 1 mgm. 4:4'-diamidino stilbene per kilo. body weight in 10 c.cm. 10 per cent. calcium gluconate. There was no reaction following the first or the subsequent three daily injections. The injections were stopped because of impending delivery.

2.2.41. Gave birth to a healthy litter.

5.2.41. Daily injections (in 10 per cent. calcium gluconate) resumed and raised to 2 mgm. per kilo. body weight. The injections were not followed by any reaction.

After 26 injections there was no change in the intensity of the infection in the popliteal gland.

5.3.41. The animal was icteric and showed anorexia.

6.3.41. The animal died.

Post-Mortem Examination. Liver enlarged and yellow. Number of parasites in popliteal gland not diminished. Numerous parasites in spleen, liver and bone-marrow. The intensive treatment to which the animal was subjected had not made the slightest impression on the infection.

5. FEMALE BOXER. Aet. 2 years. Weight 20 kilo.

Brought to the laboratory on September 29th, 1940. Depilation had been noted six months previously. On arrival in the laboratory the animal was found to have conjunctivitis. The

skin, particularly over the face, was thickened and rough. There were cutaneous ulcers in various parts. Infection in popliteal gland 20 parasites per 100 nuclei.

Between October 29th and December 18th the animal received 37 injections, each of 1.25 mgm. per kilo. The injections were not followed by any reaction.

After 37 injections the ulcers in the skin had healed, but there was no change in the intensity of the infection in the popliteal gland.

6. FEMALE BOXER. Aet. 3 years. Weight 24 kilo.

Brought to the laboratory on November 21st, 1940. Few parasites found in popliteal smear after prolonged search. History of five months' illness. The first signs noticed were seborrhoea and depilation. Later the animal developed keratitis and ulceration of the cornea and of various parts of the skin.

This case is of particular interest, since, in spite of a very slight infection, treatment with 4:4'-diamidino stilbene was a failure.

Treatment. Between January 28th and March 3rd, 1941, the animal received 28 injections of 1 mgm. per kilo. body weight 4:4'-diamidino stilbene (in 10 c.cm. of 10 per cent. calcium gluconate). No reaction after the injections.

5.3.41. Ulcers of skin healed. Smear of popliteal gland negative. Treatment stopped because of anorexia.

19.3.41. Few parasites found in popliteal gland after prolonged search.

Between March 23rd and May 23rd received another 27 injections, each of 2 mgm. per kilo. 4:4'-diamidino stilbene in 10 per cent. calcium gluconate. There were no reactions after the injections.

6.5.41. Few parasites found in popliteal gland after prolonged search.

18.6.41. Cutaneous ulcers appeared near the eyes. General condition deteriorated. Treatment commenced with urea stibamine.

Between June 18th and 30th received daily injections of urea stibamine 4 mgm. per kilo. body weight. Ulcers round the eyes improved, but treatment interrupted because of pregnancy.

7.7.41. Gave birth to a healthy litter; the organs of three puppies were examined for Leishman-Donovan bodies and found negative.

From July 29th to August 28th received 18 injections of urea stibamine, each of 4 mgm. per kilo. body weight.

28.8.41. No Leishman-Donovan bodies found in smear of popliteal gland, but there was a large number of plasma-cells. Treatment interrupted for 10 days.

Between September 8th and 20th received 11 further injections of urea stibamine, each of 4 mgm. per kilo. body weight. Smear of popliteal gland negative but contained large numbers of plasma-cells, suggesting that the infection was not eradicated (no cultures were made).

14.10.41. The animal died, probably as a result of pericarditis.

7. FEMALE BOXER. Weight 28 kilo.

Brought to the laboratory on November 17th, 1939. Blood smears showed a heavy infection with *Hepatozoon canis*. Smears from left popliteal gland negative for Leishman-Donovan bodies. Formol gel positive.

Between November 21st, 1939, and January 19th, 1940, the animal received 21 injections of 4:4'-diamidino stilbene varying from 1 to 2 mgm. per kilo. body weight. During this period a total of 0.7 gm. of the drug was given.

After the first injection the animal was unconscious for 10 minutes; the muscles of the legs and body were in a condition of tetany. There were similar reactions, occasionally accompanied by intense salivation, after each of the first 12 injections, but subsequently the animal showed no reaction after injections of 1-1.25 mgm. per kilo. body weight.

19.12.39. One Leishman-Donovan body per 100 nuclei was found in smears from the left popliteal gland. Number of *H. canis* in blood smears reduced. The animal received no further treatment after January 19th, 1940.

17.3.40. Very few *H. canis* in blood smears.

15.4.40. Smear from popliteal gland negative. No *H. canis* in blood smears.

The animal was in excellent condition and was observed for a period of two more years, during which it showed no signs of relapse. Since other animals, among them one with an even slighter infection, did not respond to much more intense treatment with 4:4'-diamidino stilbene, and since the animal was still positive after cessation of treatment, it is probable that the treatment was not the decisive factor in the animal's recovery, but that this was a case of spontaneous cure.

DISCUSSION

Although severe infections of *L. infantum* in man (Adler and Rachmilewitz, 1939; Süsskind and Roth, 1943) and light infections in Syrian hamsters (Adler and Tchernomoretz, 1941) can be cured by 4 : 4'-diamidino stilbene, we doubt whether it is possible to cure naturally infected dogs. Even after intense treatment of dogs with neostibosan or 4 : 4'-diamidino stilbene, there is a residual infection in the lymphatic glands, corneo-scleral junction and skin. The extent of the residual infection in the skin after administering a total of 1.8 gm. 4 : 4'-diamidino stilbene, and in the corneo-scleral junction after a total of 15 gm. neostibosan and 2 gm. 4 : 4'-diamidino stilbene, may be seen from the accompanying figures.

The only possible conclusion is that, until there is a further advance in the chemotherapy of visceral leishmaniasis, naturally infected dogs should be either removed to a non-endemic area or destroyed, because the residual skin-infection constitutes a source of infection for transmitting sandflies, and, through them, for other dogs and human beings.

We may add that 4 : 4'-diamidino stilbene is not a satisfactory drug for dogs. Many animals, and particularly those of the boxer breed, are sensitive and succumb after a single injection of 1 mgm. per kilo. body weight. In animals which are not susceptible, repeated injections are often followed by anorexia. Animals in this condition show minute fat-globules in the liver-cells. Anorexia is a signal for stopping the administration of the drug; if continued beyond this point the fatty degeneration becomes more pronounced.

SUMMARY

In the laboratory we have failed to cure dogs naturally infected with *L. infantum* by treatment with 4 : 4'-diamidino stilbene.

In the field we have observed failure to cure infected animals by intensive treatment with neostibosan.

After intensive treatment there is a residual infection in the corneo-sclerotic junction, lymphatic glands and skin. The latter serves as a source of infection for sandflies and, through them, for other dogs and man.

Pending a further advance in the chemotherapy of visceral leishmaniasis, infected dogs should be either removed from endemic centres or destroyed.

It is interesting to note that infected bitches produced healthy litters.

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SHOWING THE DIFFICULTY OF ERADICATING *LEISHMANIA INFANTUM* INFECTION
FROM NATURALLY INFECTED DOGS

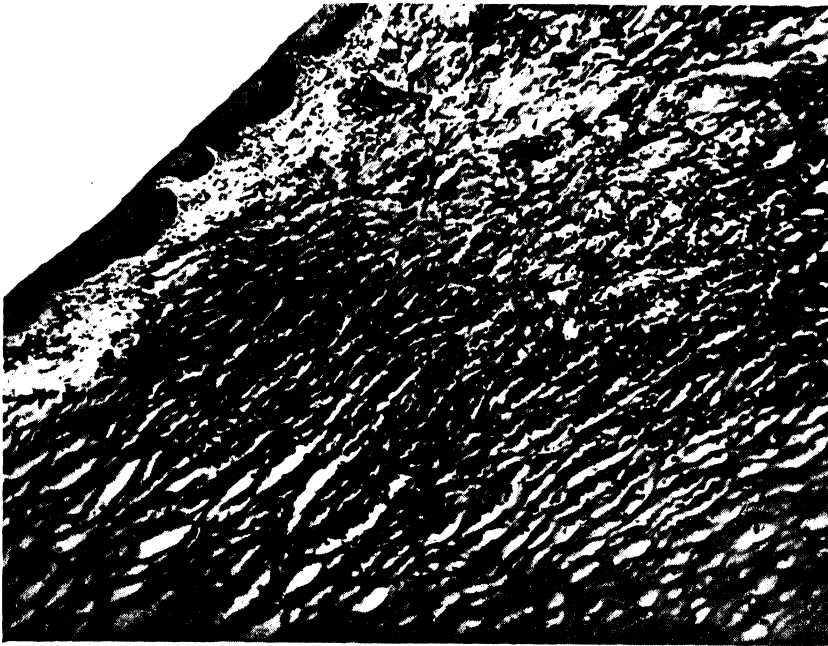


FIG. 1. Intense infiltration of the corneo-scleral junction extending into the periphery of the cornea in an animal which received a total of 15 gm. neostibosan and 2 gm. 4:4'-diamidino stilbene. The infiltration contained numerous macrophages containing Leishman-Donovan bodies. ($\times 80$.)

FIG. 2. Infected macrophage in the above infiltrate. ($\times 1,900$.)



FIG. 3. Section of the skin from the lower tarsus of an animal which received a total of 1.8 gm. 4:4'-diamidino stilbene. The skin contains numerous heavily infected macrophages. ($\times 700$.)

SNAKE SPECIES RECORDED IN THE GOLD COAST

BY

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Achimota College and the Medical Research Institute at Accra both possess good collections of snakes from all parts of the Gold Coast Colony, Ashanti, the Northern Territories and Togoland. Together with the eastern regions of the Ivory Coast, this area provides a constant snake fauna of some 74 species, as recorded at the Medical Research Institute at Accra. There are, moreover, at least 10 other species stated in the literature to be found in this area, as well as a number of unconfirmed species and varieties to be found in isolated records, none of which appears in the records at Accra, which begin in 1916.

LIST OF SNAKE SPECIES

The following snake species were collected in the Gold Coast Colony, Ashanti, the Northern Territories and Togoland during the years 1916-45. An asterisk indicates that the species has been recorded as a Gold Coast snake in the literature, but not at Accra, and that its specific rank is accepted.

TYPHLOPIDAE

Typhlops Schneider, 1801

1. *Typhlops punctatus punctatus* (Leach), 1819
2. " **caecatus* Jan, 1866
3. " **caecus* (Duméril), 1856
4. " **hallowelli* Jan, 1866

LEPTOTYPHLOPIDAE

Leptotyphlops Fitzinger, 1843

5. *Leptotyphlops brevicauda* (Bocage), 1887
6. " *bicolor* (Jan), 1866
7. " **sundevalli* (Jan), 1862

BOIDAE Pythoninae

Python Daudin, 1803

8. *Python sebae* (Gmelin), 1788
 9. " *regius* (Shaw), 1802
- Calabaria* Gray, 1858
10. *Calabaria reinhardtii* Schlegel, 1848

BOINAE

Eryx Daudin, 1803

11. *Eryx muelleri* (Boulenger), 1892

COLUBRIDAE Colubrinae

Natrix Laurenti, 1768

12. *Natrix anoscopus anoscopus* (Cope), 1861
- Neusterophis* Günther, 1863
13. *Neusterophis variegatus* (Peters), 1861
 14. " *fuliginoides* (Günther), 1858
 15. " *olivaceus olivaceus* (Peters), 1854

Chlorophis Hallowell, 185716. *Chlorophis heterodermus* Hallowell, 185717. " *irregularis* (Leach), 1819*Boaedon* Duméril and Bibron, 185318. *Boaedon fuliginosus* (Boié), 182719. " *lineatus* Duméril and Bibron, 185420. " *virgatus* (Hallowell), 1854*Grayia* Günther, 185821. *Grayia smythii* (Leach), 1818*Meizodon* Fischer, 185622. *Meizodon coronatus* (Schlegel), 1837*Philothamnus* Smith, 184023. *Philothamnus semivariatus* Smith, 1849 (or type, *Philothamnus semivariatus semivariatus* Smith, 1849)*Bothrophthalmus* Peters, 186324. *Bothrophthalmus lineatus* (Peters), 1863*Hapsidophrys* Fischer, 185625. *Hapsidophrys lineata* Fischer, 1856*Gastropyxis* Cope, 186026. *Gastropyxis smaragdina* (Schlegel), 1837*Rhamnophis* Günther, 186227. *Rhamnophis aethiopissa aethiopissa* Günther, 1862*Thrasops* Hallowell, 185728. *Thrasops occidentalis* Parker, 1940*Hormonotus* Hallowell, 185729. *Hormonotus modestus* (Duméril and Bibron), 1854*Mehelya* Csiki, 190330. *Mehelya crossii* (Boulenger), 189531. " *guirali* Mocquard, 188732. " *stenophthalmus* (Mocquard), 188733. " *poensis* (Smith), 1847*Gonionotophis* Boulenger, 189334. *Gonionotophis grantii* (Günther), 186335. " *klengi* Matschie, 1893*Lycophidion* Duméril and Bibron, 185336. *Lycophidion capense* (Smith), 183137. " *irroratum* (Leach), 181938. " *semicinctum* Duméril and Bibron, 1853(L. *irroratum* and L. *semicinctum* may be synonyms for L. *capense*.)39. " **laterale* Hallowell, 1857*Prosymna* Gray, 185840. *Prosymna meleagris* (Reinhardt), 1843

Dasypeltinae

Dasypeltis Wagler, 183041. *Dasypeltis scaber scaber* (Linnaeus), 175842. " *scaber macrops* Boulenger, 1907

Boiginae

Boiga Fitzinger, 184343. *Boiga blandingii* (Hallowell), 184444. " *pulverulenta* (Fischer), 1856*Dipsadoboa* Günther, 185845. *Dipsadoboa unicolor* Günther, 185846. " **elongata* (Barbour), 1914*Crotaphopeltis* Fitzinger, 184347. *Crotaphopeltis hotamboeia* (Laurenti), 1768 (or Cr. h. *hotamboeia*)*Tarbophis* Fleischmann, 183148. *Tarbophis variegatus* (Reinhardt), 1843*Thelotornis* Smith, 184949. *Thelotornis kirtlandii kirtlandii* (Hallowell), 1844*Dispholidus* Duvernoy, 183250. *Dispholidus typus* (Smith), 1829

Psammophis Boié, 1827

- 51. *Psammophis elegans schokari* (Forsk.) 1775
- 52. " *sibilans sibilans* (Linnaeus), 1766
- 53. " *sibilans phillipsii* (Hallowell), 1844

Dromophis Peters, 1869

- 54. *Dromophis praeornatus* (Schlegel), 1837
- 55. " **lineatus lineatus* (Duméril and Bibron), 1854

Rhamphiophis Peters, 1854

- 56. *Rhamphiophis oxyrhynchus oxyrhynchus* (Reinhardt), 1843
- 57. " *oxyrhynchus togoensis* (Matschie), 1893

Calamelaps Günther, 1866

- 58. *Calamelaps unicolor* (Reinhardt), 1843

Polemon Jan, 1858

- 59. *Polemon barthi* Jan, 1858 (probably synonymous with *Miodon gabonensis collaris*)

Miodon Duméril, 1859

- 60. *Miodon acanthias* (Reinhardt), 1860
- 61. " **gabonensis gabonensis* (Duméril), 1856
- 62. " *gabonensis collaris* (Peters), 1861

Aparallactus Smith, 1849

- 63. *Aparallactus lineatus* (Peters), 1870
- 64. " *liddiardae* Parker, 1933
- 65. " *modestus* (Günther), 1859

ELAPIDAE
Elapinae

Naja Laurenti, 1768

- 66. *Naja nigricollis* Reinhardt, 1843
- 67. " *melanoleuca* (Hallowell), 1857

Pseudohaje Günther, 1858

- 68. *Pseudohaje nigra* Günther, 1858
(And probably, rarely, *Pseudohaje goldii* Boulenger, 1895)

Dendroaspis Schlegel, 1848

- 69. *Dendroaspis viridis* (Hallowell), 1844
- 70. " **jamesoni* (Trail), 1843

Elapsoidea Bocage, 1866

- 71. *Elapsoidea güntherii* Bocage, 1866 (or *Elapsoidea sundevallii güntherii* Bocage, 1866)

VIPERIDAE
Viperinae

Atractaspis Smith, 1849

- 72. *Atractaspis dahomeyensis* Bocage, 1887
- 73. " *aterrima* Günther, 1863
- 74. " *irregularis* (Reinhardt), 1843
- 75. " *corpulenta* (Hallowell), 1854

Causus Wagler, 1830

- 76. *Causus rhombeatus* (Lichtenstein), 1823
- 77. " *lichtensteinii* (Jan), 1859

Bitis Gray, 1842

- 78. *Bitis arietans* (Merrem), 1820
- 79. " *gabonica* (Duméril and Bibron), 1854
- 80. " *asicornis* (Shaw), 1802

Atheris Cope, 1862

- 81. *Atheris chlorechis* (Schlegel), 1855 (probably *Atheris squamigera chlorechis* (Schlegel), 1855)
- 82. " *squamigera squamigera* (Hallowell), 1854
- 83. " **ceratophorus* Werner, 1895

Echis Merrem, 1820

- 84. *Echis carinatus* (Schneider), 1801

VARIATIONS OF THE EXTERNAL CHARACTERS

As mentioned above, a number of varieties within species have been recorded for the Gold Coast area in addition to those to be found in the records at Accra. These appear

to be due to variations from the typical patterns of the shields and scale formulae and of colour-patterns. When a colour-pattern is present in a species it is rare to find two individuals which can be described as identical.

The frequency of variations of shields and scales in individuals of the same species is shown by examination of the data recorded from the 456 specimens in the collection at Accra.

Form of variation	No.	Percentage of total snakes	Percentage of total variations
1. Variations from symmetrical pattern. All dorsal head-shields. (No. varying in temporal formula only = 23)	54	11.8	56.8
2. Mixed paired and single subcaudals	26	5.7	27.3
3. Symmetrical variations. Chin-shields, dorsal head-shields and anal scale	15	3.3	15.8

The total number of individuals in the above collection showing variations of the shields and scales is 95, or 20.8 per cent. It is considered that variations of the colour-pattern and external anatomy affect the nomenclature of the following species and once-reputed species.

1. *Dasypeltis scaber macrops* Boulenger.

Dasypeltis macrops Boulenger, 1907, *Ann. & Mag. Nat. Hist.*, (7), 29, 324.

The dorsal surface of the head is described as having dark-brown vermiform markings. These are disposed in the form, and are variations, of the two or three brown chevrons on the head and neck commonly seen in *D. scaber scaber* (Linnaeus), 1758, in its typical form. There is no constant arrangement of the colour-pattern of the specimens of *D. scaber* in the Accra collection.

2. *Boaedon lineatus* Duméril and Bibron and *B. fuliginosus* (Boié).

Boaedon lineatus Duméril and Bibron, 1854, *Erpet. Gen.*, 7, 363.

Boaedon lineatus lineatus Duméril and Bibron, Bogert (1940). *Bull. Amer. Mus. Nat. Hist.*, 77, 21.
Lycodon fuliginosus Boié, 1827, *Isis*, 551.

It is impossible to separate these as species by means of the descriptions in the literature, and *B. lineatus* has therefore been grouped provisionally with *B. fuliginosus* as a colour variety.

3. The genus *Lycophidion* Duméril and Bibron, 1853.

The present author has failed to find any difference of external anatomy between *L. capense* (Smith), 1831, its variety *multimaculata* Boettger, and *L. semicinctum* Duméril and Bibron. The numerical range of the scale counts of *L. capense* includes those for the others.

4. *Psammophis elegans schokari* (Forskal).

Coluber schokari Forskal, 1775, *Descr. Anim.*, 14.

Three colour varieties are found in the Gold Coast. The only difference between the varieties is in the colour of the dorsal surface of the head, which is continued in two

longitudinal lines lying one at each side of the vertebral line. Three shades of green form the basal colours. On these is superimposed a fine black stippling or speckling, which can be seen with a hand-lens. To the naked eye the dull green and black appear grey, the light green and black appear deeper green, and the dark green and black appear a delicate blue.

5. *Psammophis sibilans sibilans* (Linnaeus).

Coluber sibilans Linnaeus (part), 1766, *Syst. Nat.*, Ed. 12, 1, 323.

Boulenger's type B is the common variety. The head-markings are brownish-green rods, lines or pools, with a delicate double border. The inner border is nut-brown and the outer a thin jet black. The pattern is arranged longitudinally in the anterior of the head, but in the posterior, over the parietals, the markings are in the shape of the letter U or W. All these may be modified by shortening, narrowing or by partial or complete absence. The variety *phillipsii* has no typical *sibilans* pattern and no trace of the body lines which are so beautiful in the typical form. An undivided anal scale is a feature of the variety *phillipsii* but is occasionally seen in the type-species.

6. *Polemon barthi* Jan.

Genus *Polemon* and *P. barthi* Jan, 1858, *Rev. Mag. Zool.*, (2), 10, 520.

There may be no great difference between the colour-pattern of this species and that described for *Miodon acanthias* (Reinhardt), 1860. The two species are described in identical terms as regards their external anatomy, except for the subcaudals, which are stated to be single in *P. barthi* and paired in *M. acanthias*. A specimen in the Achimota collection with the colour-pattern of *P. barthi* has four subcaudals paired in a total of 21, and is considered to be an example of *Miodon gabonensis collaris* (Peters), 1861. A single genus would suffice to include all these closely related races.

ACKNOWLEDGEMENTS.—The author is grateful to Dr. F. J. C. Johnstone, Director of Medical Services, Gold Coast, for permission to publish this paper.

RELAPSING BENIGN TERTIAN MALARIA TREATED WITH 4430

BY

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(Received for publication April 18th, 1946)

During the first six months of 1945 trials with 4430 were carried out at Colchester Military Hospital on cases of relapsing benign tertian malaria. In all, 102 cases were treated with the drug, in doses of 900 mgm. daily for seven days. No toxic symptoms were encountered throughout the trials, and every patient completed his course of treatment. In view of the fact that renal symptoms had been observed with heavier doses of the drug, an adequate fluid-intake was insisted upon and checked by daily output of urine. At no time was there any suggestion of haematuria. Patients were allowed to get out of bed and to walk about while undergoing treatment, having been confined to bed until the temperature had remained normal for 36 hours.

Of the original 102 cases, one was found to have malignant tertian malaria and was therefore excluded from the series, and four cases could not be followed up as no response was received to repeated efforts to trace them. This left 97 cases, who were either interviewed or contacted by personal letter at the end of the six months after treatment; and, to compare with them, a series of 97 cases who had been treated with a standard course of quinine-pamaquin were followed up for an equal period. In the early part of the trials alternate cases were not treated on the two courses, but those which were chosen for the quinine-pamaquin follow-up were all treated at approximately the same time as the 4430 series.

The respective dosage and administration were as follows: 4430, 300 mgm. t.d.s. for seven days; quinine-pamaquin, quinine 10 grains and pamaquin 0.01 gm. t.d.s. for 10 days.

Relapses have been recorded as clinical or proved, and the former have been accepted only when the history was quite typical and when specific treatment was actually given. Doubtful cases have been considered as 'no relapse,' and all proved cases were shown to have parasites in the blood.

Course	Total no. of patients	No. of patients who			
		Were relapse-free	Had one relapse	Had two relapses	Had clinical relapse
4430	97	60	22	6	9
Quinine-pamaquin ...	97	81	10	1	5

This table shows the following percentage relapse-rates, including clinical relapses: on 4430, 38.1 per cent. relapsed; on quinine-pamaquin, 16.5 per cent. relapsed.

From the above figures it might at first seem that these relapse-rates are high when compared with the follow-up of 584 cases of relapsing benign tertian malaria investigated and reported upon by the statistics department of the War Office (M.L.E. 30 of the

Malaria Subcommittee of the Medical Research Council). This showed a relapse-rate of 10.3 per cent. on a six-months' follow-up on the standard 10-day quinine-pamaquin course; but it included only proved relapses, and when the present series is compared with it it is seen that, of 97 cases treated with 4430, 28 were proved to relapse (28.9 per cent.), and that, of 97 cases treated with quinine-pamaquin, 11 were proved to relapse (11.3 per cent.).

The results, therefore, on the 97 cases treated with quinine-pamaquin are almost identical with those obtained by the statistics department of the War Office referred to above, and it may be concluded that the 4430 results are equally accurate.

In regard to those cases which showed more than one relapse, it is seen that, of cases treated with 4430, 21.4 per cent. of the first relapses showed a second one, and that, of cases treated with quinine-pamaquin, 9.0 per cent. of the first relapses showed a second one.

In order to ensure that the factors involved were not influenced by the strain of the infecting parasite, the theatre of infection was compared. This shows that in the two series there was an almost identical probable area of infection.

Course	India-Burma	Mediterranean	Other areas
4430	90	6	1
Quinine-pamaquin ...	90	7	---

In view of the fact that relapses are most likely to occur in the first few months after the patient's return to the United Kingdom, and of the fact that they are often more definite and more easily recognized at that time, the interval between treatment and arrival in this country was studied, and the following figures were obtained.

Course	Average time spent in the United Kingdom (in months)	
	All cases	All relapses
4430	3.5	3.1
Quinine-pamaquin ...	2.8	2.3

The above findings would indicate that there was no bias in favour of the quinine-pamaquin course.

Analysis of the interval between treatment and relapse in the two series shows:

Course	No. of months elapsed
4430	1.9 (longest 12/52, shortest 5/52)
Quinine-pamaquin ...	2.6 (" 20/52, " 4/52)

Comparison of 50 cases of each series shows little if any difference between the two in the rapidity with which the temperature falls to normal. In no case was there failure to clear the blood of asexual parasites by the termination of the treatment.

SUMMARY

A six-months' follow-up on 97 patients treated with 4430, compared with a similar number of cases treated with the standard quinine and pamaquin treatment, showed a relapse-rate of over double that of the latter treatment. There is an indication that the interval of time between treatment and further relapse is shorter than in the quinine-pamaquin treatment; 4430 controls the symptoms of an acute attack of malaria adequately, but cannot be considered of value in the control of relapses.

ACKNOWLEDGEMENTS.—The above work was carried out at the instigation of the Malaria Subcommittee of the Medical Research Council. The author is indebted to Major-General A. G. Biggam, Consulting Physician to the Army, for permission to publish this report, and to Imperial Chemical (Pharmaceuticals) Limited, for the supplies of 4430.

THE ESTIMATION OF PALUDRINE IN URINE

BY

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AND

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(Received for publication April 23rd, 1946)

INTRODUCTION

The new antimalarial, Paludrine (Curd, Davey and Rose, 1945*b*), is N_1 -*p*-chlorophenyl- N_5 -isopropylbiguanide. A method for the estimation of this substance in biological fluids and tissues has been described by Spinks and Tottey (1945); they converted the biguanide molecule by hydrolytic fission to *p*-chloroaniline, which was then estimated colorimetrically by diazotization and coupling with a dyestuffs end component. This method is highly sensitive and capable of considerable accuracy, but it is essentially a laboratory procedure, particularly since the hydrolysis is most effectively carried out under autoclave conditions. For this reason attention has been paid in these laboratories to the discovery of simplified techniques more suitable for use in the field, and the present communication outlines a procedure much less complex than the hydrolysis method, since it requires but a few simple items of laboratory equipment. Sensitivity is considerably reduced, but is sufficient to enable accurate estimations of urine concentrations to be made in order to check excretion and hence absorption of the drug.

Biguanides are known to form co-ordination complexes with metals such as copper. Those from Paludrine are now being investigated, and their structure will in due course be recorded in detail elsewhere. It is sufficient to note here that under suitable conditions this drug forms a complex with copper which is insoluble in water but soluble in hydrocarbon solvents such as benzene, and which when isolated and characterized is found to consist of two molecules of Paludrine associated with one atom of metal and one molecule of water. The complex is feebly coloured (dull purple), unstable under acid conditions but more stable in alkali. When a solution of the complex in an organic solvent is shaken with an aqueous solution of sodium diethyldithiocarbamate, the characteristic colour of this reagent with copper is produced, the intensity of which is dependent upon the amount of copper, and hence of Paludrine, present in the test solution.

EXPERIMENTAL

The method described has been found to be satisfactory for urine containing 5-100 mgm. Paludrine per litre. Urine containing more than this amount should be diluted; if less, a larger volume may be taken, although emulsification difficulties may thereby be encountered. Acid-washed $6 \times \frac{5}{8}$ in. test-tubes fitted with rubber stoppers were used for the extraction of the copper complex into organic solvent; the rubber stoppers were well washed and boiled in distilled water after use.

Reagents

- (a) Copper reagent: copper sulphate AR (0.5 gm.) and ammonium chloride AR (1.33 gm.) dissolved in 100 ml. distilled water.
- (b) Sodium diethyldithiocarbamate: 0.1 per cent. aqueous solution. This solution should not be kept for more than a fortnight.
- (c) Normal caustic soda solution.
- (d) Benzene: the commercial grade redistilled is adequate.

Procedure

Urine (2 ml.), copper reagent (1 ml.) and caustic soda solution (1 ml.) were pipetted into a tube, mixed, and allowed to stand for a few minutes. Benzene (5 ml.) was added; the tube was stoppered and shaken for two minutes. After separation of the layers, the benzene was decanted or pipetted into a second tube, washed with 1 ml. water, transferred to a third tube and shaken with sodium diethyldithiocarbamate solution (1 ml.) for one minute. The intensity of the golden-yellow colour which developed in the benzene layer was estimated in a Spekker absorptiometer using Ilford violet 601 filters, and the amount of Paludrine present was read from a standard curve constructed from samples of urine containing known amounts of the drug.

Small separating funnels may conveniently be used for the estimation, but they may not be readily available and have been found to offer no great increase in speed and to be troublesome to clean. Xylene or chloroform may replace benzene; the commercial grade of the former may contain a volatile substance which gives a high blank reading and should be distilled over soda ash before use.

With the volumes given above emulsification has not been encountered with human urine, though it has been observed with rat urine. Such emulsions may be broken by sucking up into a capillary pipette and ejecting several times.

DISCUSSION OF RESULTS

Curve A in the accompanying diagram is a standard curve of Paludrine base in urine measured on the Spekker absorptiometer with a 1 cm. cell. A standard curve may also be obtained, with less accuracy but greater speed, using the Lovibond comparator with the appropriate disc for copper estimations with sodium diethyldithiocarbamate (curve C). Failing such an instrument, a rough estimation of an unknown sample of urine may be obtained by comparing visually the colour developed with a set of standard tubes.

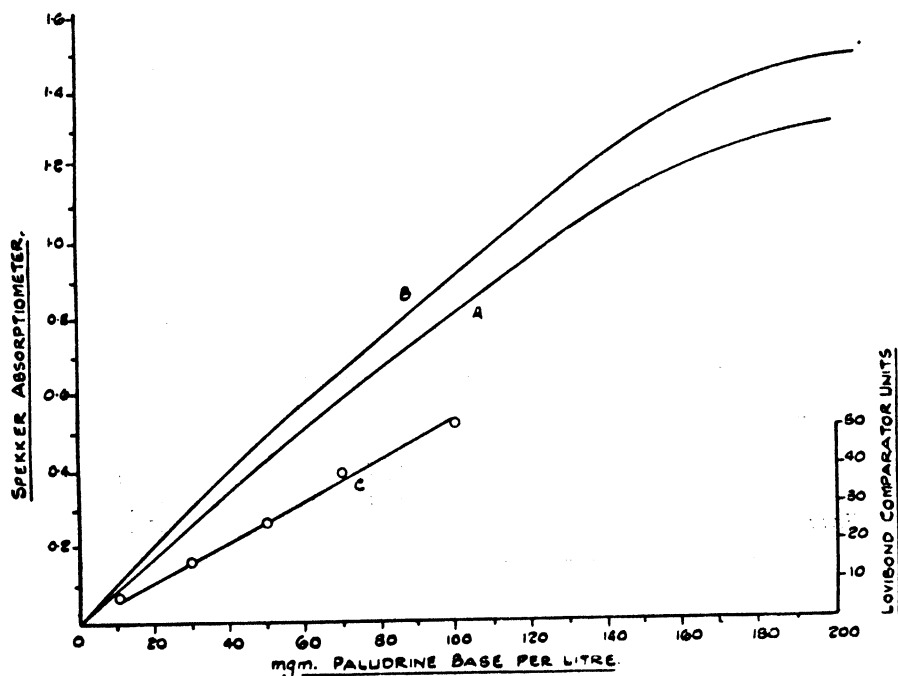
Curve B is a standard curve of Paludrine base from 0.2 M sodium phosphate solution. It will be seen that, above 100 mgm. per litre, the curves show a considerable divergence; the reason for this is not clear, and to avoid errors which may arise from variations in the salt concentration of urine it is advisable to dilute urines which contain more than 100 mgm. per litre.

A series of 10 determinations on a sample of urine containing 100 mgm. per litre show a maximum variation of ± 4.5 mgm.; the standard deviation is 2.9 mgm.

Specificity of the Method

Samples of urine were obtained from 12 normal subjects and submitted to the above procedure. Slight blank values were recorded with some, not exceeding the equivalent

of 5 mgm. Paludrine per litre. One sample of pregnancy urine and three of diabetic urine were found to give blanks not in excess of this figure. Rat urine has been found to give higher blanks, of the order of 12 mgm. per litre. These blanks do not appear to be due to some component of normal urine which forms a benzene soluble copper complex, since the same blank reading is obtained by examining the benzene extract before treatment with sodium diethyldithiocarbamate. It must, therefore, be ascribed to an extraction of urine pigment, and may be determined by reading the benzene extract of an unknown sample in the colorimeter before development of the colour due to the Paludrine-copper complex.



Derivatives of *p*-chlorophenylbiguanide with the following alkyl groups R_1 and R_2 on the terminal nitrogen atom have been examined by this method :

	Paludrine	4430	5093	4567
R_1	$C_3H_7\beta$	$C_3H_7\beta$	CH_3	$C_4H_9\beta$
R_2	—	CH_3	—	—

All were found to give colour reactions with slight variations in intensity, and the reaction is probably general for all compounds of this type ; the introduction of methyl groups into one or both of the imino-groups in the Paludrine molecule does not interfere. No colour has been obtained with biguanide, nor with *p*-chlorophenylbiguanide ; the copper complexes of these substances are presumably insoluble in benzene. Alkyl-substituted guanidine derivatives also produce no colour, nor does the antimalarial 3349 (Curd, Davey and Rose, 1945a). The method is therefore more specific than that based on the estimation of *p*-chloroaniline after hydrolysis, and it may be expected to give lower results with urine, since it may not include metabolites of the drug which give rise to *p*-chloroaniline

on hydrolysis. The experimental comparison of the two methods in general confirms this ; Table I shows the results obtained with the two methods on a series of patients undergoing Paludrine treatment at the Liverpool School of Tropical Medicine. Estimations by the hydrolysis method were performed at Liverpool by Miss M. M. Tottey.

TABLE I

Sample	Paludrine content in mgm./litre	
	Copper method	Hydrolysis method
1	202	296
2	22.7	15.4
3	412	530
4	421	531
5	17	14.25
6	190	299

Closer agreement between the two methods is noted when the urine of experimental animals dosed with Paludrine is examined. The results by the hydrolysis method were determined in this laboratory by Dr. A. Spinks.

TABLE II

Sample	Paludrine content in mgm./litre	
	Copper method	Hydrolysis method
Rat : pooled urine from 6 animals		
(a)	44.4	49.7
(b)	5.25	4.68
(c)	51.8	53.1
Rabbit	16.2	17.1

It will be noted that in both the above tables the copper method gives higher results than the hydrolysis method at lower Paludrine concentrations.

SUMMARY

A method is described for the rapid estimation of Paludrine in urine based on the extraction of the benzene soluble copper complex followed by colour development with sodium diethyldithiocarbamate.

The colour is given by mono- and dialkyl-substituted *p*-chlorophenylbiguanides, but is not given by their presumed metabolic break-down products. It is likely, therefore, to give lower results than the method based on hydrolysis to *p*-chloroaniline, and this is generally confirmed by a comparison of the results obtained by the two methods.

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MEPACRINE METABOLISM IN RECURRING BENIGN TERTIAN MALARIA

BY

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(*Received for publication May 1st, 1946*)

This paper reports the results of an investigation, made in Italy in 1944, of the metabolism of mepacrine in patients with recurring benign tertian malaria. It has already been shown that the ability of mepacrine to suppress benign tertian malaria depends on the concentration of mepacrine in the plasma (Brown and Rennie, 1946), and the plasma mepacrine concentration during treatment was therefore determined in a series of cases of malaria which had apparently 'broken through' mepacrine suppression. This series contained patients who were having their first attack and others who had had 2-11 attacks, many of them treated with mepacrine or mepacrine in combination with quinine and pamaquin; and it was found that the latter group had, on the average, lower plasma mepacrine concentrations than the former. Some possible causes of this phenomenon were then investigated.

The work was made possible by the naming of a British general hospital as a centre for the investigation of this type of case. Not only were patients with malaria sent there from other British hospitals, but men who had suffered at least five attacks of benign tertian malaria were sent between attacks from both British and Canadian hospitals for an investigation of their response to a standard course of mepacrine.

PLASMA MEPACRINE CONCENTRATIONS DURING THERAPY IN PATIENTS WITH RECURRING BENIGN TERTIAN MALARIA

Table I shows that the average plasma mepacrine concentration* found in patients who were in their fifth or later attack was lower than that in patients in their initial attack or in their second to fourth attack. As no difference in plasma level was found between those with parasitaemia and symptoms and those who were in an interval between attacks, no distinction has been made for the present purpose, and all those symptom-free at the time of the investigation have been included in the third group in Table I. The mepacrine course used in this test began with mepacrine 0.2 gm. q.d.s. for two days, and continued with 0.1 gm. t.d.s. for as long as 10 days; each dose was given by a nursing officer, who made an entry each time on a *pro-forma*. Values for days 8 and 12 are not available for the initial-attack group because their treatment course was shorter than this. Patients without parasitaemia or symptoms also received mepacrine for five days only. The difference between the average levels found on days 2 and 4 in groups 1 and 3 is statistically significant

* Mepacrine estimations were carried out by a modification of the method of Masen (1943), and specimens were collected nine hours after the last dose.

($P < 0.05$ and $P < 0.01$), and so is the difference between the average level found on day 12 in groups 2 and 3 ($P < 0.05$).

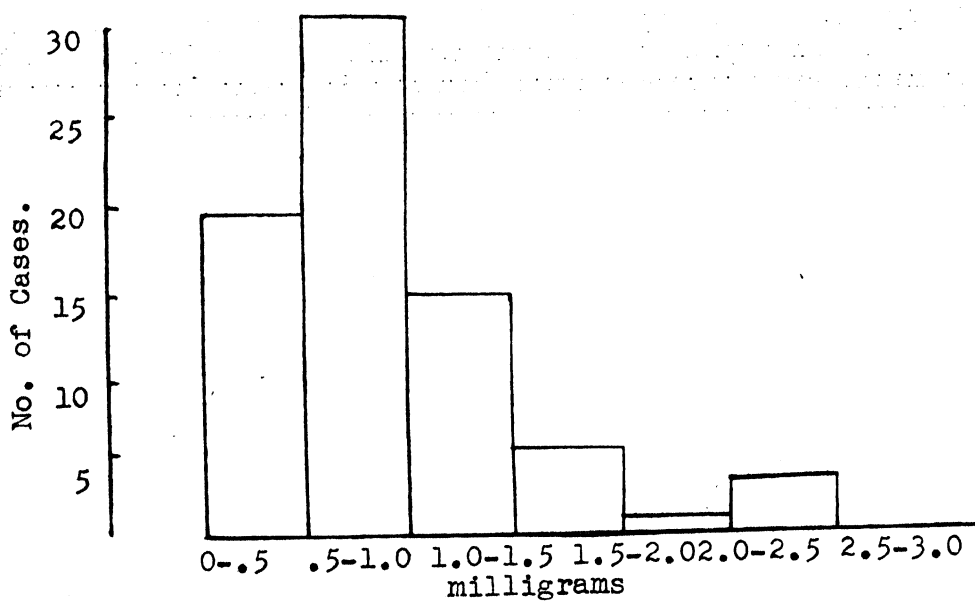
TABLE I

Average plasma mepacrine concentrations during mepacrine treatment in relation to number of previous attacks of benign tertian malaria

	Average plasma mepacrine concentrations, $\mu\text{gm.}/1,000 \text{ ml.}$			
	Day 2	Day 4	Day 8	Day 12
GROUP 1 Patients in initial attack : 13 cases 	69.0 S.E. 7.74	56.0 S.E. 5.76		
GROUP 2 Patients in 2nd-4th attack : 19 cases 	56.1 S.E. 5.60	43.8 S.E. 4.19	46.5 S.E. 4.08	44.8 S.E. 3.23
GROUP 3 Patients who had had 5 or more attacks : 42 cases 	51.5 S.E. 4.78	39.0 S.E. 2.35	36.0 S.E. 4.26 (12 cases)	33.0 S.E. 4.47 (12 cases)

EXCRETION OF MEPACRINE IN THE URINE

During the two hours immediately previous to their final venipuncture, a specimen of urine was collected from 73 cases drawn from all three groups of Table I. The mepacrine content was measured, usually by the Masen method and occasionally by a colorimetric



Mepacrine excreted in urine in 2 hours.

FIG. 1. Amounts of mepacrine excreted in the urine in two hours by 73 patients at the end of a therapeutic course.

method (King and Gilchrist, 1945). The final plasma mepacrine concentrations ranged from 5 to 100 $\mu\text{gm.}/1,000$ ml., and the amount of mepacrine excreted in the urine in two hours varied between 0.09 and 4.93 mgm. The average two-hour excretion of mepacrine was 0.94 mgm., and the frequency-distribution of the amounts is shown in fig. 1. It can be concluded that excretion of mepacrine in the urine is not the cause of the low plasma concentrations found during the treatment of recurring benign tertian malaria. The amounts excreted are obviously too small in relation to the dose of mepacrine (0.3 gm. of the dihydrochloride, 0.22 gm. of the free base) to have an important influence on the plasma concentration. Also, there is a direct correlation between the plasma mepacrine concentration and the amount in the urine ($r = 0.536$), which is the opposite of what would be expected if low plasma levels were in any way due to the excretion of large amounts of mepacrine in the urine.

LOSS OF MEPACRINE IN THE FAECES

To determine the amount of mepacrine lost in the faeces, specimens were collected from 43 cases during the last 24 hours of a therapeutic course. It was recognized that a three-day specimen would have had more value than a 24-hour specimen, but in a busy general hospital the collection of three-day specimens is very difficult and the loss of specimens is frequent. For this reason it was decided to collect 24-hour specimens and to offset the inaccuracy inherent in such specimens by studying a large series. With a series of 43 cases it is believed that a reliable figure can be given for the average daily loss of mepacrine in the faeces in groups with different average plasma levels.

The specimens were collected in 500 ml. wide-mouthed bottles, and the mepacrine content was estimated on the day of collection or within 24 hours. A suspension of the entire specimen was made by adding 250 ml. of water and a few pebbles, and by shaking for an hour. The final extraction was made from a dilution of this suspension. The final plasma levels ranged from 14 to 100 $\mu\text{gm.}/1,000$ ml., and the amount of mepacrine lost in the faeces during the last 24 hours of treatment varied from 6.65 to 128.25 mgm. The average loss was 36.8 mgm., and fig. 2 shows that there was no relation between the final plasma mepacrine concentration and the amount in the faeces. It is to be remembered when considering these figures that the amount of mepacrine is given in terms of the free base. These cases had been receiving three tablets per day for 2-9 days, which is 227 mgm. of the free base.

In some experiments done at Oxford by the Army Malaria Research Unit the amount of mepacrine in the faeces was found to be very small after the intramuscular injection of mepacrine, and it is therefore probable that the relatively large amounts found during oral therapy have not been absorbed from the gut. Failure to absorb as much as one half the daily therapeutic dose is certainly a factor which could influence the plasma level, and it is rather surprising to see in fig. 2 that there is no relation between the plasma level and the faecal mepacrine. If loss of mepacrine in the faeces were the important cause of the low plasma levels which have been found, there would be an indirect correlation between faecal and plasma mepacrine. In this series the mean 24-hour loss was 37.6 mgm. for those with plasma levels 40 $\mu\text{gm.}/1,000$ ml. or below, and 34.5 mgm. for those with higher plasma levels. With 21 cases in one group and 22 in the other, it cannot be argued that the lack of an indirect correlation was due to the collection of 24-hour rather than three-day specimens. The conclusion would seem to be that partial failure to absorb

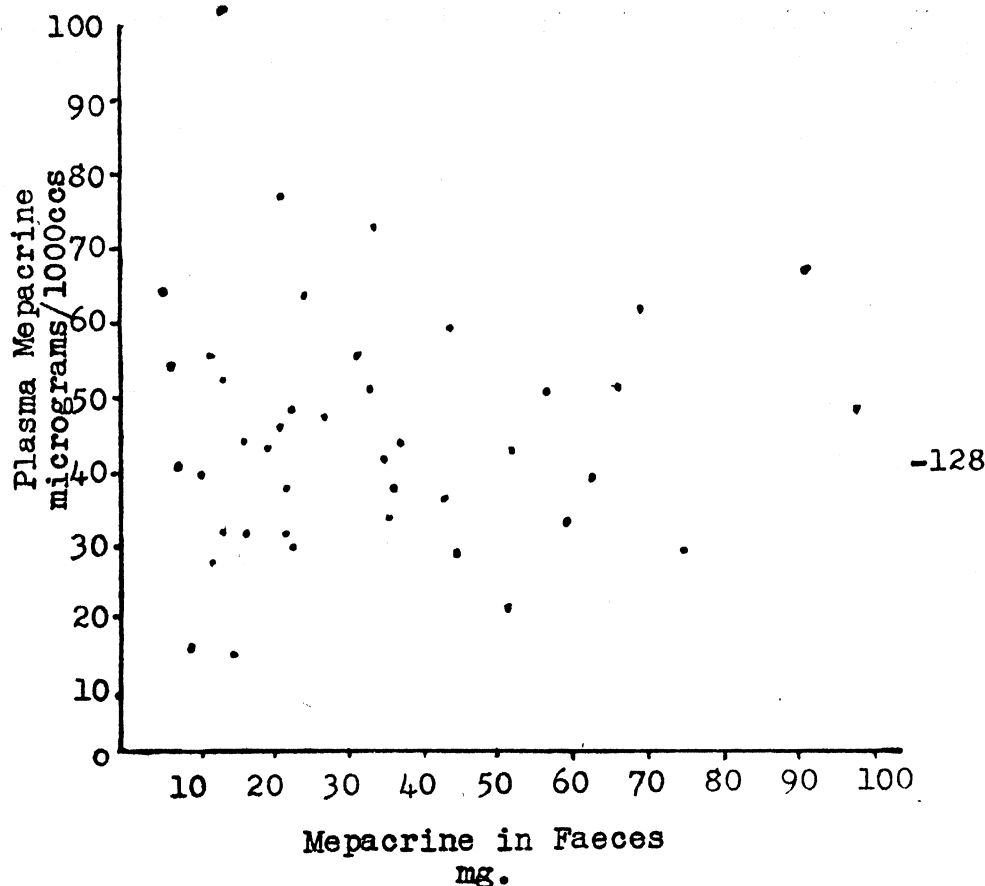


FIG. 2. Relation between the plasma mepacrine concentration and the mepacrine lost in the faeces during 24 hours of a therapeutic course.

mepacrine may contribute to the low plasma level, but that it is not the common cause and may occur in men with high plasma levels. To provide more evidence on this point, an experiment was performed in which the plasma mepacrine concentrations obtaining when mepacrine was given orally were compared with those found after intramuscular injection of the drug.

PLASMA MEPACRINE CONCENTRATIONS FOLLOWING INTRAMUSCULAR INJECTIONS OF MEPACRINE

If incomplete absorption from the gut were the cause of the low plasma levels found in relapsing benign tertian malaria, parenteral administration of the drug should be followed by an increase in the plasma levels. Plasma levels were therefore determined in a series of cases who received mepacrine by intramuscular injection on the two days following the completion of their course of oral therapy. There was not a significant increase.

The series consisted of 19 men, all but one of whom had previously had 5-10 attacks of malaria. For three or 10 days their dose of mepacrine had been three tablets (227 mgm. of the free base). In 14 cases they then received atabrine musonate by injection in a dose equivalent to 0.3 gm. of the free base. The other five cases were given 0.3 gm. of mepacrine

dihydrochloride (227 mgm. of the free base). Blood was taken for plasma mepacrine estimation 24 hours after the injection. The results of the estimation in the 14 cases who received the increased dose were corrected for the difference in dose. It is to be emphasized that the data to be presented have to do only with minimal plasma levels, i.e., the level immediately before the next dose is due, or 24 hours after the last dose, whichever is the shorter. The levels during the period of absorption have not been studied.

Table II sets forth the averaged results, and shows that there was no significant increase in plasma concentration. The increase after the first injection might have occurred by chance about once in three times, and the increase seen after two injections might have occurred by chance almost as often. Also, there was no greater tendency for the patients with very low plasma levels (less than 35 μ gm./1,000 ml.) to show an increase than there was in the group with higher plasma levels.

TABLE II
Effect of intramuscular mepacrine injection on plasma level

	Average plasma mepacrine concentration, μ gm./1,000 ml.
At end of oral therapy	34.26 ; S.E. 3.18
After one intramuscular injection	38.70 ; S.E. 4.91
After two intramuscular injections	43.61 ; S.E. 8.90

THE PARTITION OF MEPACRINE BETWEEN CELLS AND PLASMA

It is well known that the concentration of mepacrine in the tissues is much higher than it is in the plasma. Concentrations in the plasma are given in μ gm./1,000 ml., whereas in the tissues mepacrine is given in mgm./100 gm. This fact has led to the hypothesis that low plasma levels may be due in some cases to a partition between tissues and fluids which is different from the average. The hypothesis has been tested, and the results suggest strongly that a difference in the partition of mepacrine between tissues and fluids is not the explanation of the low plasma levels in cases of recurring benign tertian malaria.

Methods. Blood was taken from 20 cases of relapsing benign tertian malaria on completion of mepacrine therapy, and the following determinations were made :

1. Plasma mepacrine concentration.
2. Whole-blood mepacrine concentration.
3. Mepacrine concentration of packed red cells.
4. White blood-cell count.
5. Haematocrit.

The amount of mepacrine contained in the white-cell layer was calculated by subtracting from the whole-blood mepacrine concentration the amounts contributed by the plasma and the red cells. This figure was corrected for a white-cell count of 10,000/c.mm. before it was compared with the plasma mepacrine concentration. By multiplying the corrected figure by 100 an approximation to the concentration in the white cells can be obtained. At first the white-cell mepacrine was determined directly, using the waisted-tube method of separating white cells ; but the results of the two methods were in good agreement, and the indirect method was finally chosen because it was less laborious. The mepacrine in the platelets has been neglected, because it is relatively small compared with the amount in whole blood. The results are given in Table III.

Relation Between Plasma and White-Corpuscular Mepacrine. In fig. 3 the plasma mepacrine concentration has been plotted against the mepacrine content of the white-cell layer after correction for any variation in the white-cell count from 10,000 c.mm. There is a direct correlation of surprising degree ($r = 0.79$; for 20 cases, $P = 0.05$, when $r = 0.44$) throughout a range of plasma levels from 14 to 75 $\mu\text{gm.}/1,000 \text{ ml.}$ It was also found that there is a direct relation between the mepacrine concentration of the red-cell layer and the plasma mepacrine.

TABLE III

Mepacrine concentrations in plasma and blood-corpuscles at the end of a therapeutic course of mepacrine

Case no.	Mepacrine concentrations, $\mu\text{gm.}/1,000 \text{ ml.}$			Haematocrit	Mepacrine content of white cells in 1,000 ml. blood	White-cell count
	Plasma	Whole blood	Red cells			
342	53	532	384	44	332	11,600
343	75	400	192	52	264	8,400
344	56	312	176	44	205	5,850
352	36	240	184	49	132	11,540
354	14	72	64	43	36	7,850
356	46	200	176	39	203	4,600
357	46	360	464	41	143	5,850
358	31	240	224	42	128	9,000
360	61	728	328	45	546	14,250
361	53	608	912	41	212	12,350
362	15	108	56	42	75	10,050
363	40	252	216	48	127	9,150
365	59	240	96	44	165	7,800
369	23	148	40	50	116	12,550
370	38	204	96	47	139	7,500
376	39	212	88	49	149	10,100
377	39	248	104	52	191	12,850
380	27	156	104	44	94	8,250
381	49	164	176	52	97	12,550
387	70	432	144	53	323	10,750

Changes in Mepacrine Content of the Corpuscles. If specimens are taken at short intervals after a single dose of mepacrine, some interesting facts can be learned about the partition of mepacrine between corpuscles and plasma. Two men who had not had mepacrine for at least three months were each given eight tablets at one time, and specimens were taken $\frac{1}{2}$, 1, 2, 4, 8, 12 and 24 hours later. They were then given four doses of two tablets during the second 24 hours, followed by one tablet t.d.s. for eight days, which corresponded to the course of mepacrine in common use. Specimens were taken each morning before the first dose, so that the changes in mepacrine content of the corpuscles could be followed throughout the course. The results are shown in Table IV.

During the period of absorption there is a rapid increase in the mepacrine content of the white cells, and the white-cell mepacrine/plasma mepacrine ratio becomes very much higher than it is at the end of 24 hours. In the post-absorptive period the white-cell mepacrine/plasma mepacrine ratio falls, and the amount of mepacrine given up during these hours is comparatively great. In case 1 this amount was 840 $\mu\text{gm.}$, which was more than nine times as much mepacrine as was circulating in the plasma (assuming a plasma volume of 3,000 ml.) 24 hours after the administration of the tablets. The white-cell

TABLE IV

Mepacrine concentrations in plasma and blood-corpuscles during a therapeutic course of mepacrine

	Mepacrine concentrations, $\mu\text{gm.}/1,000 \text{ ml.}$			Haematocrit	Mepacrine content of white cells in 1,000 ml. blood	White-cell count
	Plasma	Whole blood	Red cells			
CASE 1						
$\frac{1}{2}$ hour	9	32	48	46	5	5,750
1 "	9	—	—	—	—	—
2 hours	44	132	88	45	25	8,700
4 "	60	200	176	45	88	8,900
8 "	65	268	144	44	168	10,350
12 "	42	208	144	44	87	9,950
24 "	29	132	—	—	—	—
3rd day	63	212	200	44	89	7,650
4th "	69	256	216	43	124	7,950
5th "	46	220	208	43	104	6,850
6th "	66	268	224	45	131	8,950
7th "	59	316	176	45	205	8,650
8th "	56	284	152	47	185	8,900
CASE 2						
$\frac{1}{2}$ hour	10	28	32	47	8	10,100
1 "	19	76	40	46	49	9,550
2 hours	50	304	88	45	236	10,600
4 "	34	268	104	47	201	9,750
8 "	18	176	72	48	131	10,950
12 "	16	176	88	47	122	11,350
24 "	32	128	88	47	65	10,250
3rd day	38	232	—	—	—	—
4th "	58	328	224	44	152	11,250
5th "	51	420	160	45	321	11,400
6th "	45	268	120	45	192	9,050
7th "	56	298	160	44	198	9,150
8th "	53	234	108	46	132	5,600
9th "	37	256	140	44	158	9,250

mepacrine/plasma mepacrine ratio at the time when the plasma level is minimal increases from day to day up to about the fifth or sixth day, and then remains relatively constant.

The evidence suggests that in the period of absorption the circulating white cells pass through a medium with a higher mepacrine concentration than there is in the peripheral plasma, and that in the post-absorption period much of the mepacrine which they have taken up in the previous period is returned to the plasma and distributed to other tissues. The mepacrine which is returned to the plasma is apparently lightly bound by the white cells. During the post-absorption period following the first dose, the white-cell mepacrine/plasma mepacrine ratio falls to a level which is lower than the minimal level after the fifth day, which could not occur if all the mepacrine in the white cells at the peak concentration during absorption were held in the same manner as the mepacrine in the white cells when the plasma level is minimal. Evidently mepacrine is bound in the white cells in two ways: the lightly bound mepacrine increases and decreases rapidly after a dose is given; the firmly bound mepacrine increases more slowly and reaches a constant value for a given plasma level about the fifth day, when the dosage is 800 mgm. for two days and then 300 mgm. per day.

Discussion. Before conclusions are drawn from these experiments, it is necessary to consider the suitability of the white blood-cells as an example of the body-tissues

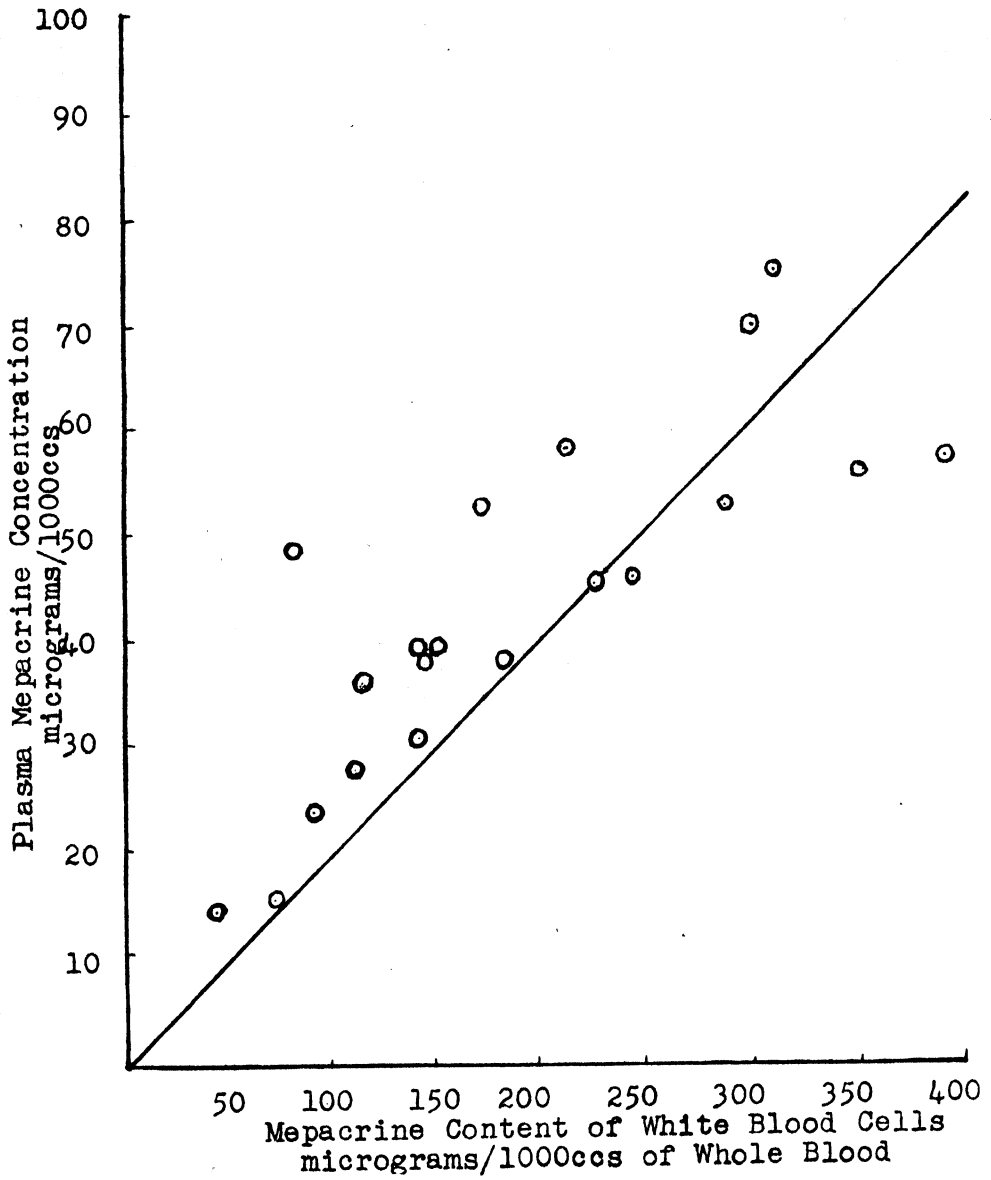


FIG. 3. Relation between mepacrine content of plasma and white blood-cells at the end of a therapeutic course.

generally. The technical reasons for the choice are obvious: the white cells are the only tissue from which it is possible to take repeated biopsies. There are several other reasons for accepting them as a fair sample for the study of the metabolism of mepacrine. The facts that they have a relatively short life in the peripheral circulation, that they develop from the endothelial system, and that they have a nucleus and an active protoplasm need not be stressed. It is known that they take up mepacrine to the same degree as organs such as the spleen and lung, and it has now been shown that they behave like the body

generally in that an equilibrium concentration is gradually reached when mepacrine is given daily. These are the considerations which supported the choice of the white cells for these experiments.

The direct correlation which has been found by comparing the plasma mepacrine concentration with the white-cell concentration at the end of treatment is too strict to permit the argument that the reason why some of the plasma levels were as low as 14 or 15 $\mu\text{gm.}/1,000$ ml. was that the tissues in these cases contained proportionally more mepacrine than the tissues in the cases with high plasma levels. If this were the reason, the relation would be an indirect one, and instead it is a direct one with a surprisingly high coefficient of correlation. The cause of the great variation of plasma levels seen in a group on the same dosage of mepacrine must be sought elsewhere.

CONCLUSIONS

It has been demonstrated in terms which are statistically significant that men with relapsing benign tertian malaria show low plasma mepacrine concentrations during a standard mepacrine course. The fact that men without parasitaemia or symptoms at the time of the test had levels which were as low as those who were in an attack disposes of the improbable suggestion that the levels were low because of the disease. It seems, rather, that the men who show low plasma concentrations during treatment are prone to relapses. A relatively low plasma concentration is sufficient to cause the parasitaemia to disappear, but apparently a high concentration is more effective in ridding the body of infection.

There are four possible causes of the low plasma levels shown by some men on a standard course of mepacrine: the drug might be excreted in abnormally large amounts in the urine; it might not be absorbed from the gut; the partition between tissue and plasma might be abnormal; or the drug might be destroyed abnormally rapidly, so that the equilibrium concentrations are low both in plasma and in tissues. The evidence of the experiments described rules out the possibility of an abnormally large excretion through the kidneys being the responsible factor. The observations of the faecal excretion of mepacrine and of the plasma mepacrine concentration after intramuscular administration of a dose which previously had been given orally do not support the suggestion that failure of absorption is responsible, though they do not exclude the possibility in a minority of cases. The partition between cells and plasma has been shown to be constant. This leaves by exclusion differences in the rate of destruction of mepacrine as the common cause of variation of plasma concentrations from the average.

Would mepacrine be a more effective drug in the treatment of benign tertian malaria if a high plasma level—say, about 60 $\mu\text{gm.}/1,000$ ml.—were achieved in every case? On the evidence it would seem more than possible. The problem of administering the drug in such a way that a high level is obtained is not likely to be difficult, for the levels found in this series after mepacrine 1.6 gm. had been given in two days show that high levels can be obtained when the dose is sufficiently large. In individual cases the plasma mepacrine concentration may be determined with sufficient accuracy by the indirect method which we have already described (Brown and Rennie, 1945), and, if mepacrine is used in the treatment of relapsing benign tertian malaria, it might be valuable to control the dosage in this way.

SUMMARY

1. It was found in Italy in 1944 that patients with recurring attacks of benign tertian malaria treated on a standard course of mepacrine showed, on the average, lower plasma mepacrine concentrations than did patients in their first attack.

2. Observations during treatment on patients with recurring benign tertian malaria showed that the low plasma mepacrine concentrations were not caused by abnormal excretion of the drug in the urine or by an abnormal partition between tissues and plasma, and that they were not commonly due to a failure of absorption from the gut.

3. It was concluded that the common cause of low plasma concentrations during treatment was an abnormally rapid destruction of the drug by the body, and that, if it is to be used in the treatment of relapsing benign tertian malaria, the plasma mepacrine concentration might with value be controlled by observations on the urine.

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A SEROLOGICAL ANALYSIS OF TYPHUS CASES IN INDIA BY WEIL-FELIX, RICKETTSIAL AGGLUTINATION AND COMPLEMENT-FIXATION TESTS

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INTRODUCTION

The early writings on typhus in India, which are reviewed by Covell (1936a), make it clear that the classical epidemic disease was recognized from the close of the last century onwards as occurring in outbreaks in those parts of northern India where the term 'cold weather' expresses a fact and not a mere trifling diminution of the heat. Megaw (1917) was the first to suggest that forms of typhus other than louse-borne occurred in India, when he described his own illness, occurring three weeks after a tick bite, as 'a case of fever, resembling Brill's disease.' Four years later (Megaw, 1921), he correlated this illness with a number of others reported by McKechnie (1913) from the same neighbourhood, and, on an analogy with Rocky Mountain spotted fever coupled with his own experience, suggested that they were 'possibly transmitted by ticks.' From these beginnings the expression Indian tick-typhus has come into currency. The term is a convenient one, which we shall use in this paper, but it should always be remembered that the rôle of the tick as a vector of typhus in India awaits proof. By 1934 the Weil-Felix reaction had come into general use in the military laboratories of India, and in the next year Boyd (1935) published an analysis of 110 cases of fever of the typhus group which he separated into three categories according as the agglutinins in the patients' sera were predominantly to *Proteus* OX19, OX2 or OXK. The OX19 group was further subdivided on clinical grounds into two sections, of which one was indistinguishable from the OX2 group (the Ahmednagar-Poona type), and the other (the Bangalore type) was suggested as being endemic or murine typhus, which was subsequently shown by Covell (1936b) to occur in India. Thus the position was arrived at where on serological grounds it was possible to separate mite-typhus from the rest, but not to distinguish with certainty between louse-, flea- and tick-typhus, as in the last either the OX2 or OX19 agglutinins might predominate.

Of recent years additional methods of approach to the problem have been afforded by the production of high concentrations of rickettsiae from infected mouse lungs (Castaneda, 1939) or chick-embryo yolk-sacs (Cox, 1938) from which agglutinable rickettsial suspensions have been prepared in the cases of epidemic and murine typhus, and complement-fixing rickettsial antigen in these and in Rocky Mountain spotted fever, *fièvre boutonneuse*, Q fever and scrub typhus.

With regard to the relative specificity of rickettsial agglutination and complement-fixation, Plotz, Bennett, Wertman and Snyder (1944) reported that, while murine rickettsiae were agglutinated by Rocky Mountain spotted fever sera, there was no cross-fixation of

complement. On the other hand, Fitzpatrick (1945) found no agglutination of epidemic or murine rickettsiae by sera of rabbits hyperimmunized to Rocky Mountain spotted fever. Bengtson (1945) found that of 216 Rocky Mountain spotted fever sera 92.1 per cent. gave no cross-fixation with murine antigen, and of 114 murine sera 80.7 per cent. gave no cross-fixation with Rocky Mountain spotted fever antigen. Those sera which did give cross-fixation showed a much higher titre with the homologous antigen. Plotz, Wertman and Reagan (1944) pointed out the value of complement-fixation in places where several varieties of typhus occur, and gave examples of its specificity in murine and epidemic typhus and in Rocky Mountain spotted fever as opposed to the Weil-Felix test. Plotz, Reagan and Wertman (1944) differentiated between *fièvre boutonneuse* and Rocky Mountain spotted fever by complement-fixation. Rickettsial agglutination allows of a distinction being drawn between murine and epidemic typhus, as shown by van Rooyen and Bearcroft (1943) in Cairo, but their table contains several cases with a high OX2 titre, which might for that reason have been called tick-typhus, diagnosed as murine or epidemic typhus on the basis of high agglutinins to the corresponding rickettsiae. In sum, it would seem to be clear that the complement-fixation test is more specific and of wider application at present than the rickettsial agglutination reaction.

It is evident that the application of these more specific tests to the study of the typhus group of fevers in India would help to define their relationship to rickettsial diseases elsewhere in the world. Topping, Heilig and Naidu (1943) reported that the sera from three cases of typhus in Mysore showed strong complement-fixation with Rocky Mountain spotted fever antigen and low-titre cross-fixation with murine and, in one case, epidemic antigen, which cross-fixation, they pointed out, was unusual and difficult to explain. In this paper we have applied complement-fixation, rickettsial agglutination and Weil-Felix tests to a number of typhus sera from various parts of India, and have tabulated the results on the assumption that the complement-fixation reaction is the most specific of the tests.

MATERIALS AND METHODS

Since August, 1944, a directive has been in force requiring the dispatch to our laboratory of sera from all military personnel suffering from typhus fever in India. Most of the specimens received were from patients in the third or fourth week of the illness, and serial specimens were unfortunately rare. The Weil-Felix agglutination test, originally performed at the various hospital laboratories, was repeated, and rickettsial agglutination tests using murine and epidemic suspensions were done on all those showing an X19 or X2 response. Complement-fixation tests, with murine typhus and Rocky Mountain spotted fever antigens, were performed on a number of the more recent specimens. The sera were received preserved by sodium merthiolate, 1 part in 10,000. The Weil-Felix and rickettsial agglutination tests were done so soon as these specimens were received, but the complement-fixation tests were done in some instances on sera that had been stored for varying periods at 4° C. The effect of this will be discussed later.

Weil-Felix and Rickettsial Agglutination Tests

The antigens employed for the Weil-Felix test were standard army suspensions of *Proteus* OX19, OX2 and OXK. These concentrated suspensions were diluted 1 in 15 in sterile normal saline before use. The rickettsial suspensions were prepared from the

infected lungs of mice or rats by the method described by Fulton and Begg (1946). The strains used were the Naples epidemic and the Mysore murine strains, which originally were obtained lyophilized from Major Janet Niven, R.A.M.C., of the Emergency Vaccine Laboratory, Everleigh, Wiltshire.

Before use, the rickettsial suspensions were diluted with sterile normal saline to match in opacity the concentrated standard army *Proteus* OX19 suspension diluted 1 in 20. Agglutination tests were set up in quintuplicate in Dreyer's tubes, using 0.3 ml. of serial dilutions of the serum for testing (1 in 50 to 1 in 12,800 or higher), and 0.3 ml. of suspensions of *Proteus* OX19, OX2, OXK, and epidemic and murine rickettsiae. The mixtures were incubated in a water-bath at 37° C. for 18 hours before reading. The highest dilution showing standard agglutination was taken as the end-point.

The Complement-Fixation Tests

The technique was adapted from that recommended by Bengtson (1944). Two antigens were used. The rat-lung suspension of murine rickettsiae used in the rickettsial agglutination test was also used as the murine antigen in the complement-fixation tests. It was found that the dilution employed for rickettsial agglutination also gave the most satisfactory results for complement-fixation. Even in 10 times the concentration used for testing, this antigen had no anti-complementary activity. The Rocky Mountain spotted fever antigen, prepared from infected yolk-sacs, was sent to us by Dr. Cox, of Lederle Laboratories Incorporated, New York. This antigen was anti-complementary before dilution but not in a dilution of 1 in 2. In the actual tests dilutions of 1 in 3 or 1 in 6 were used. Both murine and Rocky Mountain spotted fever antigens were stored at 4° C. and diluted immediately before use.

'Lyovac' pooled guinea-pig complement was reconstituted and titrated immediately before each batch of tests. Using 2 M.H.D., as in Bengtson's technique, many sera were anti-complementary, so 3 M.H.D. of complement were used throughout, which possibly accounts for the rather low titres obtained.

The sera to be tested were all inactivated at 56° C. for half an hour. Because of the relative thermolability of the *Proteus* agglutinins several sera were examined for the effect of heat on the complement-fixing antibody. After two hours at 56° C. the titre fell only slightly. An example is shown in Table I.

TABLE I
Showing the effect on the complement-fixation titre of varying the time of desensitization of the serum
Serum 347 versus murine antigen

Minutes at 56° C.	Reciprocals of dilution						Control
	10	20	40	80	160	320	
30	4	4	4	4	4	2	0
60	4	4	4	4	4	2	0
90	4	4	4	4	4	1	0
120	4	4	4	4	3	1	0

4 signifies complete fixation.

0 signifies no fixation.

1, 2 and 3 signify intermediate degrees of fixation.

The haemolytic system consisted of 3 per cent. washed sheep's red cells, which were mixed with an equal volume of 3 M.H.D. haemolysin and incubated in the water-bath at 37° C. for 30 minutes for complete sensitization.

For the test proper the inactivated serum was put up in duplicated serial dilutions from 1 in 5 to 1 in 320 in 0.125 ml. amounts. To each tube was added 0.125 ml. of 3 M.H.D. complement followed by an equal quantity of the appropriate murine or Rocky Mountain spotted fever antigen. Fixation was carried out for one hour in the 37° C. water-bath. Ice-box fixation was tried, but it was found that after this the Rocky Mountain spotted fever antigen was anti-complementary, even in high dilutions.

After fixation 0.25 ml. of sensitized red cells was added to all tubes, which were then incubated at 37° C. for a further 30 minutes, when the results were read.

The following controls were always used:

1. Anti-complementary control of serum: 1 in 5 serum, 0.125 ml.; complement, 0.125 ml.; saline, 0.125 ml.; sensitized red cells, 0.25 ml.
2. Haemolytic control of serum: 1 in 5 serum, 0.125 ml.; complement, 0.125 ml.; saline, 0.125 ml.; unsensitized red cells, 0.25 ml.
3. Anti-complementary control of antigen: antigen, 0.125 ml.; complement, 0.125 ml.; saline, 0.125 ml.; sensitized red cells, 0.25 ml.
4. Haemolytic control of antigen: antigen, 0.125 ml.; complement, 0.125 ml.; saline, 0.125 ml.; unsensitized red cells, 0.25 ml.

Since standard guinea-pig sera were not available as positive and negative controls, two patients' sera, which had constantly shown strong fixation against the two antigens respectively, with no cross-fixation, were used instead. The titre of the serum was taken as the highest dilution showing 3 or 4 plus fixation.

RESULTS

During the 18-months period from September 1st, 1944, to March 1st, 1946, sera were received from 216 patients. Sixty-nine of these were from cases of scrub typhus, and in these no investigation was done apart from confirmation of the isolated high OXK titre. The remaining 147 sera were examined by the Weil-Felix and rickettsial agglutination tests. A representative selection of results is given in Table II.

TABLE II
Showing the Weil-Felix and rickettsial agglutination results in 12 typhus sera

Case no.	Nationality	Area of infection	Day of disease	Weil-Felix			Rickettsial agglutination	
				X19	X2	XK	Epidemic	Murine
8	Indian	Bannu	24	200	50	50	200	800
55	"	Deolali	41	400	25	25	200	1,600
57	British	Poona	7	640	25	100	640	640
			17	400	—	—	400	3,200
70	Indian	Delhi	22	6,400	125	50	400	3,200
149	"	Aurangabad	16	6,400	50	50	6,400	25,600
179	African	Calcutta	9	1,600	200	50	1,600	6,400
76	Indian	Mehgaon (C.P.)	14	100	800	25	100	800
80	British	Budni (C.P.)	24	400	800	25	200	1,600
145	"	Delawari (C.P.)	29	25	100	100	100	800
151	Indian	Assam	28	25	400	25	200	400
127	"	Bairagarh (C.P.)	31	100	800	100	100	400

It was found that in a few cases murine and epidemic rickettsiae were agglutinated to an equal titre, which never exceeded 1 in 640, but in the great majority of instances agglutinins to murine rickettsiae were predominant, and this occurred whether the *Proteus* agglutinins were principally to OX19 or OX2, from which it followed that the rickettsial agglutination reaction did not help in distinguishing between murine and tick-typhus.

Complement-fixation tests were done on 40 typhus sera received during the last six months of the period under review. As in no case did the epidemic rickettsial titre exceed the murine, it seemed probable that all sera were from either murine typhus or members of the tick-typhus group, the latter with an indefinite agglutination reaction, but possibly showing an immunological relationship with Rocky Mountain spotted fever by the complement-fixation technique. This supposition was in fact confirmed.

In 18 cases complement was fixed in the presence of murine rickettsial suspension, with no cross-fixation (Table III). In 16 cases fixation occurred in the presence of Rocky Mountain spotted fever antigen, with low-titre cross-fixation in one case (Table IV). In five cases the test was negative, and in one fixation occurred to an equal titre with both antigens (Table V).

As mentioned above, complement-fixation tests were also done on 16 sera showing high OX2 agglutinins, all of which had been stored at 4° C. for periods exceeding eight months. Of these, only four fixed complement—two in the presence of Rocky Mountain spotted fever antigen and two with murine rickettsiae. This sharp contrast with the results obtained with fresh sera was taken to be due to deterioration of serum during storage, and these findings are not further considered.

TABLE III
Eighteen cases showing complement-fixation with murine suspension
(All titres expressed as reciprocal of dilution)

Case no.	Nationality	Area of infection	Day of disease	Weil-Felix			Rickettsial agglutination		Complement-fixation	
				X19	X2	XK	Epidemic	Murine	R.M.S.F.	Murine
278	British	Poona	18	<50	2,500	50	100	800	Nil*	40
291	"	Dacca	{ 14	3,200	50	50	400	6,400	"	20
			23	1,600	50	50	800	1,600	"	40
309	Indian	Deolali	21	1,600	100	25	<200	1,600	"	80
315	"	Roorkee	18	1,600	<50	60	800	6,400	"	>320
320	"	"	12	1,600	1,600	—	800	3,200	"	>320
334	"	Bannu	20	800	<50	<50	<200	3,200	"	20
340	"	Deolali	20	400	200	<50	200	800	"	40
341	"	"	18	200	3,200	—	<200	800	" *	>320
347	"	Rawalpindi	40	1,600	400	50	200	6,400	"	160
355	"	Bangalore	21	400	200	<50	<50	1,600	"	40
362	"	Jhelum	—	800	25	—	<200	1,600	" *	80
363	"	Bannu	17	500	250	<50	200	1,600	" *	>320
364	"	Agra	14	1,600	400	50	400	1,600	"	>320
367	"	Bangalore	—	100	800	—	100	100	"	40
369	British	"	26	800	200	<50	200	3,200	"	20
377	Indian	Bannu	25	800	<50	<50	<200	400	"	20
378	"	Jhelum	44	3,200	—	—	200	—	" *	40
380	"	Jullundur	29	3,200	<50	<50	200	3,200	" *	160

*Rocky Mountain spotted fever antigen at 1 in 6.

TABLE IV

Sixteen cases showing complement-fixation with Rocky Mountain spotted fever suspension
(All titres expressed as reciprocal of dilution)

Case no.	Nationality	Area of infection	Day of disease	Weil-Felix			Rickettsial agglutination		Complement-fixation	
				X19	X2	XK	Epidemic	Murine	R.M.S.F.	Murine
296	British	Comilla	33	50	800	<50	<200	200	5	Nil
305	Indian	Bangalore	16	50	400	50	<200	400	160	"
312	British	"	21	200	1,600	—	100	200	5	"
333	Indian	Bannu	19	800	<50	<50	<200	1,600	5	"
338	"	Bairagarh	16	<50	200	—	100	200	>320*	"
339	"	"	22	<50	800	—	<50	200	160	"
342	"	Jhansi	37	100	400	400	<200	<200	20	"
349	British	Bangalore	16	<50	800	100	<50	400	10	"
350	Indian	Amritsar	12	3,200	800	<50	400	400	5	"
351	Italian	Poona	18	100	1,600	50	<20	<20	Nil*	—
			100	<50	<50	<50	<50	<50	10	Nil
360	Indian	Bannu	26	3,200	400	50	200	1,600	20	"
368	"	Bangalore	15	50	1,600	—	400	400	10	"
373	"	Chindwara	35	50	200	100	100	400	160	20
374	"	"	28	50	400	<50	100	200	80	Nil
379	British	Bangalore	17	1,600	<50	50	200	200	40*	"
381	"	"	21	400	800	—	200	200	80*	"

* Rocky Mountain spotted fever antigen at 1 in 6.

TABLE V

Six cases showing equivocal or no complement-fixation
(All titres expressed as reciprocal of dilution)

Case no.	Nationality	Area of infection	Day of disease	Weil-Felix			Rickettsial agglutination		Complement-fixation	
				X19	X2	XK	Epidemic	Murine	R.M.S.F.	Murine
306	British	Bangalore	15	400	1,600	—	<200	400	Nil	Nil
316	African	Bairagarh	—	100	6,400	—	400	6,400	"	"
361	Indian	Bannu	27	400	<200	<50	<200	400	5	5
365	"	Agra	14	1,600	50	<50	400	3,200	Nil	Nil
370	"	Bannu	26	800	<50	<50	<200	400	"	"
372	British	Bangalore	24	800	<50	<50	50	400	"	"

The titres obtained were, on the whole, low. Only six were higher than 1 in 320, and four were only 1 in 5. The reason for this, as mentioned above, was possibly the use of 3 M.H.D. of complement. The higher dilution of Rocky Mountain spotted fever antigen did not produce significantly lower titres. According to Bengtson (1945) even titres as low as 1 in 4 are significant, as examination of sera from a large number of normal people and patients suffering from non-rickettsial diseases were completely negative by her technique. In order to confirm the significance of low-titre fixation, a small series of control-tests was performed on 37 sera, the nature of which is shown in Table VI.

All the tests were negative at a dilution of 1 in 5. It was, therefore, assumed that even low-titre fixation was good evidence of rickettsial infection with the homologous or closely related strain.

TABLE VI

Distribution of control-cases giving negative complement-fixation tests with Rocky Mountain spotted fever and murine typhus antigens

Type of case	No.	Type of case	No.
Normal (British)	11	Acute rheumatism	1
" (Indian)	9	Epilepsy	1
Scrub typhus	7	Chronic bronchitis	1
Salmonella infections	3	Lobar pneumonia	1
Small-pox	2		
Pulmonary tuberculosis	1	Total	37

Relationship Between the Three Tests

There was generally a close correlation between the tests, inasmuch as in tick-typhus sera the *Proteus* OX2 agglutinins usually exceeded the OX19 and the murine rickettsial titres were relatively low, while in murine typhus the reverse obtained (Table VII). Nevertheless, in individual cases the non-specificity of the Weil-Felix and rickettsial agglutination reactions was demonstrated. Thus in three cases of murine typhus the *Proteus* agglutination was of the OX2 type, and in four cases of tick-typhus it was of the OX19 type. Similarly, in two of the tick-typhus group strong murine rickettsial agglutination occurred.

TABLE VII

Showing the relationship between the type of typhus and the *Proteus* and rickettsial agglutinations

		No. of cases	
		Murine typhus	Tick-typhus
<i>Proteus</i> agglutinations	{ OX19 titre > OX2 titre	15	4
	{ OX19 titre < or = OX2 titre	3	12
Murine rickettsial agglutinations	{ 1 in 800 or higher	15	2
	{ 1 in 400 or lower	2	14

There was no relationship between the actual titre of complement-fixation and that of rickettsial agglutination or Weil-Felix in these results. Nevertheless, in two patients with endemic typhus, not included in the series, from whom specimens of serum were obtained at varying intervals, there was a relationship between the complement-fixation and rickettsial agglutination titre but not the *Proteus* OX19 titre (figs. 1, 2). These two cases also demonstrated an unusually early appearance of the Weil-Felix agglutinins, which maintained a fairly steady high level from the first week, whereas the rickettsial agglutinins and complement-fixing antibodies rose to a peak in the fourth week. Case no. 291 (Table III) also showed the later rise of complement-fixing antibodies. Unfortunately in case no. 351 (Table IV) the dilutions of antigens used were different on the two occasions, but there was fixation after three months, when there was no longer any agglutination with *Proteus* OX2.

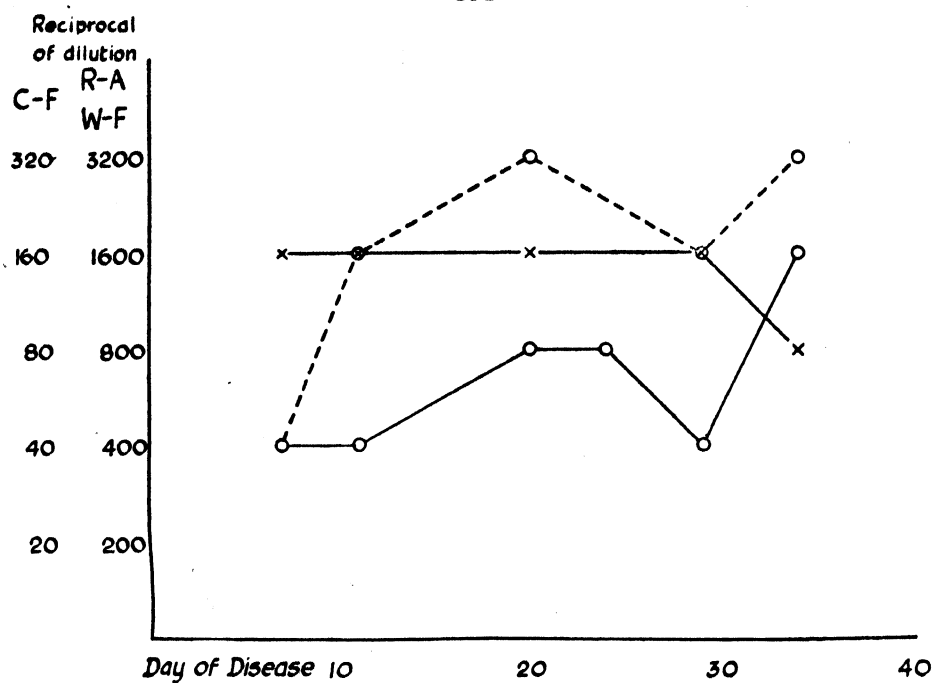


FIG. 1

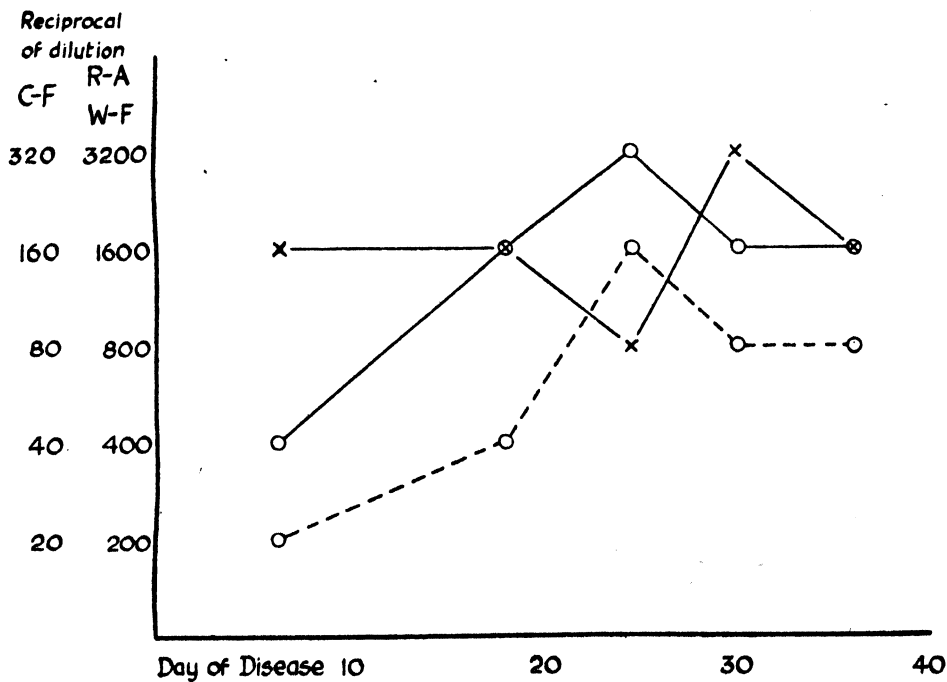


FIG. 2

FIGS. 1 and 2. Showing the relationship between complement-fixation, murine rickettsial agglutination and *Proteus* OX19 titres in two cases of murine typhus.

○—○—○ = Complement-fixation (C-F).
 ○---○---○ = Rickettsial agglutination (R-A).
 ×—×—× = *Proteus* agglutination (W-F).

DISCUSSION

Indian Tick-Typhus

It is apparent that 16 patients were infected with a strain of rickettsiae antigenically similar to the causative agent of Rocky Mountain spotted fever. Among the pathogenic rickettsiae generally there is a close relationship between the antigenic structure and the arthropod vector. As Rocky Mountain spotted fever can only be transmitted by ticks, the complement-fixation tests furnish further indirect evidence that tick-borne typhus occurs in India. These 16 cases correspond to Boyd's (1935) OX2 group and to his 'Ahmednagar-Poona' section of OX19 group.

The three sera from Mysore examined by Topping evidently fell into the same category, though the prominent cross-fixation with murine antigen observed by him did not occur in our series, where cross-fixation was present in one instance only. In view of the finding of Plotz, Reagan and Wertman (1944) that Rocky Mountain spotted fever and *fièvre boutonneuse* can be distinguished by complement-fixation, it would be interesting to examine Indian tick-typhus sera by complement-fixation with *fièvre boutonneuse* antigen, but the true position of Indian tick-typhus will not be known until antigens have been prepared from locally isolated strains. One such strain was isolated in our laboratory (case 351, Table IV), but experimental work with it was prevented by its low virulence, which diminished rapidly with successive passages in guinea-pigs and mice. It is also necessary to study the tick as the probable vector. Heilig and Naidu (1944) failed to isolate strains from cattle-ticks, and so far no strains have been isolated in our laboratory from dog-ticks (*Rhipicephalus sanguineus*).

Kalra (1946) isolated a strain of rickettsiae from unidentified ticks taken from a rat at Palel, a hyperendemic focus of scrub typhus near the Burma border. From the rat itself a typical strain of *R. tsutsugamushi* was obtained in mice, but from the ticks an entirely different strain was isolated. The ticks were inoculated into a guinea-pig, which 12 days later developed fever and a scrotal reaction. It was killed on the 16th day and passaged to two more guinea-pigs. Three further passages were made, all guinea-pigs developing fever and dying, but only two showing a scrotal reaction. The guinea-pig of the fifth passage survived without developing signs of infection. It was brought to our laboratory, where its serum was found to fix complement in the presence of Rocky Mountain spotted fever antigen (4 plus at 1 in 10 and 2 plus at 1 in 20 serum dilutions). No fixation occurred with epidemic, murine or scrub typhus antigens. A *Macacus rhesus* monkey, also inoculated at the fifth passage, after a six-days' incubation-period developed fever lasting five days. Serial Weil-Felix tests showed a rise in OX2 agglutinins after 43 days. (The late rise of OXK agglutinins in monkeys infected with scrub typhus has also been noted.) Complement-fixation tests could not be done as the monkey's serum was anti-complementary.

Other Varieties of Indian Typhus

The widespread occurrence of murine typhus in India is already well known. Several strains have been isolated, and the flea has been shown to be the vector and the rat the reservoir, as in other parts of the world. That no cases of epidemic typhus occurred in military personnel during the period under review was no doubt in part due to the higher standard of hygiene enjoyed by the army as compared with the civilian population, among whom there is no lack of the necessary vector; but it is a curious fact that epidemic

typhus in general is less common in the tropics than might be expected. In India it seems to be confined to winter outbreaks among the civilian population in the subtropical north, and even there it never assumes such serious proportions as, for example, in Egypt. Scrub typhus is probably widespread throughout the jungles of India. The bulk of our sera came from cases of the disease contracted on the Burma front, where the population at risk was greatest; but a considerable outbreak occurred in a training establishment at Gudalur in the Mysore jungle, and isolated cases developed in such places as Jullundur in the Punjab and Bandipura in Kashmir.

The Complement-Fixation and Rickettsial Agglutination Tests in Diagnosis

The rickettsial agglutination reaction is at present limited in its application to distinguishing epidemic and murine typhus in places where these are the only forms of typhus occurring. If agglutinable suspensions of rickettsiae other than *R. prowazeki* and *R. mooseri* can be prepared, the usefulness of the test will doubtless be extended. A wider range of antigens with a higher degree of specificity is available for the complement-fixation reaction, which is consequently the method of choice at the present time. The chief obstacle to the more extended use of these tests in the diagnosis of typhus is not their technical difficulty, which is hardly greater than that of a Widal or Wassermann reaction, but the difficulty and expense of preparing the antigen, both of which are considerable and likely to prevent the tests from being used as a routine diagnostic measure save in a reference laboratory.

SUMMARY

1. The sera from cases of the typhus group of fevers in India were examined by Weil-Felix reaction, rickettsial agglutination (using epidemic and murine rickettsiae) and complement-fixation (using Rocky Mountain spotted fever and murine rickettsial antigens), and the results were classified on the assumption that complement-fixation was the most specific of the three tests.
2. Scrub typhus sera could easily be recognized by their isolated high titre to *Proteus* OXK, and were not further examined.
3. It was shown that while in general murine typhus sera agglutinated *Proteus* OX19 to a higher titre than *Proteus* OX2, and the reverse obtained in 'tick-typhus,' the Weil-Felix reaction did not afford an absolute means of distinguishing between these diseases.
4. Murine rickettsiae were agglutinated by both murine typhus sera and tick-typhus sera, usually to a higher titre in the former case than in the latter, but no absolute distinction could be drawn.
5. Forty sera agglutinating murine rickettsiae and either or both *Proteus* OX19 and OX2 were examined by complement-fixation. Of these, 18 fixed complement in the presence of murine rickettsiae with no cross-fixation, 16 did so in the presence of Rocky Mountain spotted fever antigen, with cross-fixation in one case only, and that to a much lower titre, while of the remaining six sera five were negative with both antigens and the sixth was positive with both to a titre of 1 in 5.
6. It is concluded that the 'tick-typhus' of India is antigenically similar to Rocky Mountain spotted fever, and that this affords some indirect evidence that 'tick-typhus' is in fact carried by ticks.

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INVESTIGATIONS IN THE CHEMOTHERAPY OF MALARIA IN WEST AFRICA

V.—SULPHONAMIDE COMPOUNDS *

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The efficiency of high initial doses of mepacrine in controlling malignant tertian malaria in West Africa has already been demonstrated (Findlay, Markson and Holden, 1944a, 1944b).

In the present communication the effect of certain sulphonamide compounds in the treatment of malignant tertian malaria in European personnel in West Africa is recorded.

The compounds investigated were :

Sulphadiazine	18 cases
Sulphathiazole	18 cases
Succinylsulphathiazole	10 cases
Sulphamezathine (sulphamethazine)	40 cases
Sulphapyrazine	20 cases
Sulphamerazine	20 cases
† W.F. 301 (the cinnamylidene Schiff's base of diaminodiphenyl sulphone with four sulphonic acid groups)	10 cases

The following dosage was employed. Succinylsulphathiazole : 18 gm. for six days—a total of 108 gm. W.F. 301 : 5 gm. for three days, 4 gm. for two days, 2 gm. for one day—a total of 25 gm. The remaining drugs were all used in the following dosage : 6 gm. on the first day and 4 gm. daily for a further five days—a total of 26 gm.

The experimental conditions and the personnel involved were similar to those described when comparing the therapeutic effects of mepacrine and quinine. Parasites were always present in the peripheral blood-stream at the time of commencing treatment. Thick blood films were examined morning and evening.

The effect of sulphonamide compounds on the plasmodia of man, animals and birds has been fully reviewed by Curd (1943). The greater number of the recorded observations on human malaria has been carried out on infections due to either *Plasmodium vivax* or *P. malariae* induced for therapeutic purposes. Certain sulphonamide compounds have, however, been previously tested on *P. falciparum* infections. These compounds are prontosil rubrum, prontosil soluble, sulphanilamide, rubiazol, proseptazine, soluseptazine (p,p'-diaminodiphenyl sulphone N,N'-didextrosesulphonate), sulphapyridine and sulphadiazine. All these compounds had some action in controlling fever, although with

* Summary of a report submitted in 1944 to the Malaria Committee of the Medical Research Council but not published for security reasons.

† For a supply of this compound we are indebted to the Wellcome Foundation. The others were donated by the United States Malaria Commission.

prontosil soluble and soluseptazine the action was very slight. In removing trophozoites from the peripheral blood, prontosil rubrum, prontosil soluble and sulphanilamide appeared to be less active than the others. Apart from a questionable action exerted by rubiazole (Durand, 1939), none of the compounds had any action on gametocytes.

Up to the present, no experiments appear to have been carried out with West African strains of malignant tertian malaria, and, of the compounds used by us, only sulphadiazine has previously been studied elsewhere. Coggeshall, Maier and Best (1941) found that, of five patients with infections due to *P. falciparum*, two were unaffected by the drug, parasites and fever still continuing; in the other three, parasites were removed from the peripheral blood in one, two and two days, while fever disappeared in three, one and one days respectively.

Sulphadiazine has been used by Johnson (1943) in induced infections due to *P. malariae* and *P. vivax*—13 of the former and one of the latter; fever and parasites duly disappeared, but three patients had relapses, which were controlled by a further course of the drug.

Sulphathiazole has not been used against *P. falciparum*, but in *P. vivax* infections Schwartz *et al.* (1941) found that of nine patients treated no parasites could be found in the blood of five at the end of the treatment; one of these, however, relapsed 15 days later; the blood of the remaining four patients continued to harbour parasites, and they had relapses in 12, 13, 16 and 20 days respectively after the termination of treatment.

RESULTS

Duration of fever and of parasites in the peripheral blood, following the commencement of administration of the drugs, was selected as the most reliable index of therapeutic effect. Duration of symptoms for the most part ran parallel to the duration of fever.

DURATION OF FEVER

Detailed results of the duration of fever are shown in Table I. Considering the drugs in the following order, sulphathiazole, sulphamezathine, sulphadiazine, succinylsulphathiazole, sulphamerazine, W.F. 301 and sulphapyrazine, the average duration of fever following the commencement of each drug was 48, 55, 60, 72, 86, 89 and 101 hours respectively.

DURATION OF PARASITES IN THE PERIPHERAL BLOOD

Relationship between the duration of fever and the duration of parasites in the peripheral blood was as follows:

TABLE I

Drug	Average duration of fever, in hours	Average duration of parasites, in hours
Sulphathiazole ...	48	63
Sulphamezathine ...	55	62
Sulphadiazine...	60	58
Succinylsulphathiazole	72	79
Sulphamerazine ...	86	68
W.F. 301 ...	89	16
Sulphapyrazine ...	101	96
Quinine ...	63	25
" and mepacrine	60	41
Mepacrine 0.3 gm. ...	75	67
" 0.6 " ...	64	63
" 0.8 " ...	66	48

The marked discrepancy between the two figures in the case of W.F. 301 is explained by the low initial parasite counts in the series. Prolonged fever in cases of malaria with low parasite counts suggests that the drug is of questionable value.

The salient points in regard to the duration of parasites may be summarized as follows :

SULPHADIAZINE

In one only of the 18 cases was there an initial parasite count of more than 100 per 10 fields. While the average duration of parasites in the blood was 58 hours, the variation was from 12 to 156 hours. In six cases the parasite count, which had risen after 36 hours' medication, was not below the initial level till 60 (in three cases), 72, 84 and 108 hours after beginning therapy. On the whole there was a moderate response to this drug. Two cases failed to respond, and quinine was used.

SULPHATHIAZOLE

Three cases had an initial parasite count of more than 100 per 10 fields. In three cases with low initial counts, parasites lingered on in the peripheral blood for 108 hours or more, and in one of these quinine had to be used after 120 hours of sulphonamide therapy. The average duration of parasites in the peripheral blood was 63 hours, variations being from 12 to 156 hours, apart from the case placed on quinine. Of the 18 cases only one showed a parasite rise after 36 hours' medication.

SUCCINYLSULPHATHIAZOLE

The average duration of parasites in the peripheral blood was 79 hours, with variations of from 24 to 216 hours. One case had an initial parasite count of more than 100 per 10 fields; counts in the remainder were low, varying from 2 to 36 per 10 fields. Parasites were still present after 216 hours in one case, and in two others quinine had to be used after 72 hours, as neither parasites nor symptoms were controlled. In the remaining seven cases the parasite count had decreased after 36 hours, except in two cases.

A further case, not included in the series, in which succinylsulphathiazole was used without effect is of interest since it is an example of a double infection with *P. falciparum* and *P. ovale*. The patient had served in Egypt and Palestine in 1935-37, but had not knowingly suffered from malaria before arrival in West Africa. He had taken 0.6 gm. mepacrine weekly for one and a half months before exhibiting a clinical attack of malaria. On the second day of fever *P. ovale* was found in the blood. As the temperature had shown no tendency to fall by the evening of the third day, and as symptoms were becoming severe and malignant tertian parasites were still present in the blood, quinine grains 10 was given intravenously, and mepacrine 0.3 gm. daily.

This drug is obviously of little value, as might be expected in view of its poor absorption from the intestine. Unfortunately it was not possible to estimate blood-levels.

SULPHAMEZATHINE (Sulphamethazine)

The average duration of parasites was 62 hours, with variations of from 12 to 120 hours. Four cases had parasite counts above 100 per 10 fields; one case showed 1,200 parasites in 10 fields: parasites had disappeared in this patient in 108 hours. Nine cases showed parasite rises after 36 hours' medication. The drug was ineffective in three of

40 cases, and mepacrine and quinine had to be used. No toxic results were observed from the use of mepacrine immediately following sulphamezathine. In one patient the parasites, which had disappeared after 24 hours' heavy infection (314 parasites per 10 fields), returned after 96 hours, rising to 700 per 10 fields.

TABLE II
Occurrence of trophozoites and gametocytes following sulphamezathine therapy

No. of case	Trophozoites : no. of hours in peripheral blood	Day of appearance of gametocytes	Duration of gametocytes, in days
1	24	2	47 +
2	84	5	31 +
3	24	1	27
4	48	4	13
5	60	4	14
6	24	0	44 +
7	108	2	25
8	24	6	20
9	12	4	23
10	12	2	40 +
11	36	3	13
12	36	1	50 +
13	84	2	22
14	84	4	54 +
15	48	1	13
16	36	1	5
17	24	0	—
18	108	4	10
19	60	7	26
20	36	0	—
21	24	0	—
22	108	5	35 +
23	84	0	—
24	24	0	—
25	72	5	8
AVERAGE	47.3	3.4	

+ = Still present on last day examined.

SULPHAPYRAZINE

This drug proved to be of little value, and some of the cases provided very picturesque temperature-charts, resembling those due to *P. vivax* infections. In three cases the drug was completely ineffective in controlling either parasites or fever, and infection was terminated by quinine or mepacrine after 108, 120 and 132 hours on sulphapyrazine medication. The average duration of parasites was 84 hours, with variations of from 24 to 132 hours. Six of the 16 patients had initial parasite counts of over 100 in 10 fields, while six cases showed an increase in the parasite count after 36 hours of medication.

SULPHAMERAZINE

There was no response in four cases out of 20, and quinine or mepacrine had to be used after from 120 to 192 hours. The average duration of parasites in the 16 cases that responded was 68 hours, varying from 12 to 108 hours. Three patients had initial parasite counts of more than 100 per 10 fields—2,400, 800 and 330 respectively ; three cases showed a rise in parasite count after 36 hours of medication. It is of some interest that in the

four cases which did not respond the highest initial count was 60 in 10 fields, and in only two was there a rise to 800 and 250 parasites after 84 and 60 hours respectively.

W.F. 301

While the rapid disappearance of parasites may be associated with an initial low parasite count, it is noticeable that in only one of the 10 cases was there a rise in the parasite count, and in no case was it necessary to resort to quinine or mepacrine. From recent experience it is not impossible that some of these cases would have remitted without treatment.

DURATION OF PALPABLE SPLEEN

It is of interest to compare the length of time for which the spleen remained palpable in the sulphonamide-treated cases with that in the series treated with quinine and mepacrine (Findlay, Markson and Holden, 1944a).

Drug	Average duration of palpable spleen, in days	Combined average
Mepacrine 0.3 gm. ...	4.4 days	4.5 days
" 0.6 " ...	6.0 "	
" 0.8 " ...	3.7 "	
Quinine ...	4 "	
Sulphadiazine ...	9.3 days	8.4 days
Sulphathiazole ...	7.2 "	
Sulphamerazine ...	7.5 "	
Sulphamezathine ...	8.3 days	8.4 days
Sulphapyrazine ...	11.3 "	
Succinylsulphathiazole ...	6.7 "	
W.F. 301 ...	3.5 " (2 cases only)	

This comparison gives further evidence of the relative inefficiency of the sulphonamide drugs in controlling infection compared with quinine and mepacrine.

RELAPSE-RATE

A recrudescence of symptoms and fever and the reappearance of parasites in the blood occurring after the termination of treatment and while the patient was still in hospital were taken to indicate relapse rather than reinfection, since the patients were nursed in mosquito-proofed wards.

The following table shows the relapse-rate in cases treated by the drugs under investigation.

Drug	No. of cases	No. of cases relapsing
Sulphathiazole ...	18	3
Sulphadiazine ...	18	2
W.F. 301 ...	10	1
Sulphamerazine ...	20	1
Sulphamezathine ...	40	1
Sulphapyrazine ...	20	0
Succinylsulphathiazole ...	10	0

Such relapses have seldom been observed by us in cases treated with quinine or mepacrine.

TOXIC REACTIONS

Deep cyanosis was observed in all the cases treated with W.F. 301 and eight of the patients complained of headache. No other toxic effects were noted. Sulphamerazine was responsible for the only other toxic reactions. Of a total of 20 cases, two developed renal colic, with microscopic blood and crystals of acetylsulphamerazine in the urine, while a third patient developed complete anuria following renal colic and gross haematuria.

Complete anuria has not previously been recorded with sulphamerazine, but Callomon (1944) has recently found that this compound is more likely to produce urinary deposits experimentally in rabbits than either sulphathiazole or sulphadiazine. A full description of this case of anuria is given in the appendix to the present paper.

EFFECT ON GAMETOCYTES

A notable phenomenon in the sulphamezathine-treated series was the tendency for showers of crescents to appear in the peripheral blood both during and after treatment. Observation on a considerable number of cases treated with quinine or mepacrine has shown that approximately only 15 per cent. show gametocytes in the blood, usually in from 8 to 15 days after commencing treatment, and that the numbers of gametocytes are not large. Occasionally, however, after mepacrine showers of gametocytes are seen in the peripheral blood, but, as shown in Table II, 19 of 25 patients treated with sulphamezathine produced gametocytes in the peripheral blood in an average of 3.4 days. Of the 19 gametocyte-carriers seven showed one or more gametocytes per field. It appeared of interest to determine, since the drug was obviously not directly gametocidal, whether the gametocytes of patients given sulphamezathine were still capable of infecting *Anopheles gambiae*.

The experiments were carried out in collaboration with Captain J. D. Robertson, R.A.M.C., who bred the mosquitoes in the laboratory from larvae collected in the Gold Coast.

Four Europeans, who had been given sulphamezathine 26 gm. in six days for treatment of an attack of malaria and who were found to have high gametocyte counts, were bitten by laboratory-bred *A. gambiae*.

The results were as follows :

Patient	No. of days after beginning treatment with sulphamezathine	Gametocyte concentration	Mosquito infectivity	
			Positive	Negative
S.M. 1 ...	40	1 in 20 fields	0	5
S.M. 7 ...	30	1 " 3 "	0	6
S.M. 11 ...	28	1 " 1 "	0	5
S.M. 22 ...	18	1 " 5 "	0	7

These results did not actually prove that the crescents had been damaged by the sulphamezathine, which had, apparently, induced them. Since all the patients, after

taking their course of 26 gm. of sulphamezathine in six days, had been given mepacrine 0.6 gm. weekly as a suppressive, they had been on mepacrine for 34, 24, 22 and 12 days respectively before being bitten. It is obvious that the mepacrine rather than the sulphamezathine may have damaged the gametocytes and rendered them incapable of infecting *A. gambiae*.

It was therefore necessary to use patients who were not taking a suppressive drug. Three adult Africans with gametocytes in their blood were bitten by laboratory-bred *A. gambiae* and were then placed on a course of 26 gm. of sulphamezathine in six days; at intervals they were again bitten by mosquitoes.

The results of the experiments were as follows :

Patient	No. of days after beginning treatment	Gametocyte-rate	Mosquito infections	
			Positive	Negative
W	0	2 in 50 fields	12	7
	4	2 " 50 "	3	4
	13	2 " 50 "	0	9
X	0	3 in 50 fields	8	4
	13	1 " 40 "	0	4
	17	2 " 50 "	0	12
P	0	3 in 50 fields	4	9
	8	1 " 50 "	0	9

By combining the three experiments it will be seen that before treatment, of 44 mosquitoes given a potentially infective meal, 24 became infected as shown by the development of oöcysts in the stomach; after the patients had had a course of sulphamezathine for six days, of 34 mosquitoes given a potentially infective meal, none became infected.

DISCUSSION

Although far less effective than either mepacrine or quinine, in the dosage used the sulphonamide compounds tested had some action in removing the plasmodia of malignant tertian malaria from the blood-stream and in reducing fever and symptoms. The action of succinylsulphathiazole and sulphapyrazine was, however, slight.

With sulphadiazine, sulphathiazole, sulphamezathine and sulphamerazine the action was slightly more marked. A small proportion of the very mild cases possibly recovered spontaneously.

In assessing the value of any new antimalarial compounds in the treatment of malaria it is necessary to take into account the fact that, in Europeans who have taken suppressive mepacrine (0.6 gm. a week) for a considerable time, some clinical attacks due to *P. falciparum* remit spontaneously without further treatment beyond a purge and rest in bed.

Since there is some evidence that the sulphonamides act on malarial parasites by replacing *p*-aminobenzoic acid—a mechanism almost certainly different from that of mepacrine and quinine—it is possible that the more active sulphonamides might be of value in occasional cases where quinine and mepacrine have failed to control fever or to remove parasites from the blood, or where there is idiosyncrasy to these drugs. The following cases are illustrative of this point.

M., medical officer, had been in West Africa for six months, during which time he had five attacks of malignant tertian malaria. Courses of mepacrine and quinine, alone or in combination, though reducing fever, failed to remove parasites from the blood for more than 48 hours. He was given sulphamezathine 26 gm. in six days. By the third day parasites had disappeared from the blood; they were absent for eight weeks. A course of mepacrine 2.8 gm. in six days was then given, and the patient has since remained free of symptoms—a period of six months.

Mrs. H. had been in West Africa for 18 months, during which time she had had seven attacks of malaria. Mepacrine upset her, producing nausea and vomiting, while quinine caused deafness. She was given 26 gm. of sulphamezathine in six days, followed by 0.5 gm. sulphapyrazine daily as a suppressive, and remained free from malaria for the next five months.

The action of the sulphone, W.F. 301, is of interest, although in all the cases the initial parasite count was low. In only one case, however, was there a rise in parasite count following medication, and in this case parasites had disappeared after 60 hours.

The failure of sulphapyrazine to control either parasites or fever is of some interest, as there is evidence that it has some suppressive action (Findlay, 1944).

It is possible that the dosage of the sulphonamide compounds was too small, and that better results might be obtained with larger doses; but the occurrence of urinary complications in three cases of the sulphamerazine series is a reminder that great care must be observed in using sulphonamides in Europeans in the tropics, especially in a disease such as malaria, where profuse sweating and subsequent loss of fluid is common. A high fluid-intake must be maintained, and the urine must be examined frequently in all cases of malaria treated with sulphonamides.

The few experiments with sulphamezathine on crescent-carriers suggest that the infectivity of the gametocytes may be damaged by this drug; but no conclusion can be drawn until further experiments have been carried out.

SUMMARY

The therapeutic effect of certain sulphonamide drugs has been investigated in cases of malignant tertian malaria in European troops in West Africa.

Sulphadiazine, sulphathiazole, sulphamezathine and sulphamerazine had a slight effect in controlling infections, but were far inferior to mepacrine and quinine.

Sulphapyrazine and succinylsulphathiazole were of little value. Nineteen of 25 cases treated with sulphamezathine showed gametocytes in the blood films. There is some evidence that such gametocytes are incapable of infecting *A. gambiae* mosquitoes.

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APPENDIX

Corporal H. W., aged 25, had been in West Africa for 18 months. He had had two attacks of malaria, the last in December, 1943, when he received 26 gm. of sulphamerazine in six days. During this attack he had some abdominal discomfort, vomiting and diarrhoea, while the urine contained a trace of albumin but no red blood-corpuscles.

In the evening of January 15th, 1944, he vomited three times, but the next day he felt better. At night, however, he had a rigor, and on the following day he felt feverish and vomited. On examination the temperature was found to be 104° F., pulse 102, respiration 20; the tips of the spleen were tender and just palpable; the edge of the liver was just palpable and tender. A blood film showed malignant tertian rings.

18.1.44. Sulphamerazine 6 gm. (1 gm. every four hours).

19.1.44. Sulphamerazine 4 gm. (1 gm. every six hours). Spleen tender, 2 in. below left costal margin.

20.1.44. No symptoms; weak. Sulphamerazine continued: 4 gm. in 24 hours.

21.1.44. At 21.00 hours on the previous night he complained of abdominal colic, and in the next five hours passed six watery stools. 04.00 hours: severe pain in the right loin, continuing for the next six hours. During the night urine was passed normally, but between 08.00 and 12.00 hours only 90 ml. of dark-brown urine was passed. Microscopically, the urine contained numerous erythrocytes and dumb-bell-shaped crystals.

Sulphamerazine was stopped after a total of 14 gm. had been given. The patient vomited frequently and had intense pain radiating from the kidneys to both inguinal regions. Morphia $\frac{1}{2}$ grain was given. Intravenous glucose saline (5 per cent.) was injected, three pints up to 22.00 hours. No urine was passed. The bladder was cystoscoped and a number of small flame-shaped sub-mucous haemorrhages were seen. Whitish debris was present on the mucosa in the region of the trigone. No urine apart from 3 c.cm. of almost pure blood was in the bladder, and no urine was seen issuing from the reddened ureteric orifices. The right ureter admitted a catheter for 4 cm., the left ureter for 6 cm. Obstruction was thought to be due to spasm. Four ml. of sterile water were injected into each ureter: from the right ureter the water at once flowed back into the bladder, but on the left side it passed up to the kidney pelvis and at once caused colic.

22.1.44. 08.30 hours: during the previous 12 hours only 2 oz. of deeply blood-stained fluid were passed per urethram; the patient vomited several times. B.P. 110/50. Blood urea 55 mgm. per 100 ml. The ureters were again catheterized: the right was still blocked, but the left allowed the passage of the catheter and clear urine drained at the rate of 1 drop per second, 60 ml. being collected in 12 minutes. The left catheter was left in position, and 215 ml. of urine with a gritty sediment were passed. After 14 hours the catheter became blocked, but after being syringed the flow recommenced.

The catheter was removed after 24 hours. Intravenous glucose continued. Malignant tertian rings were found in the blood film.

24.1.44. The patient vomited during the night; complete anuria. Blood urea 145 mgm. per 100 ml. The subcutaneous tissues were becoming oedematous. On examining the bladder at 22.00 hours both ureteral orifices were oedematous; the bladder mucosa was inflamed and oedematous. The right ureter would admit a catheter for only 2 cm.; the left ureter allowed the passage of the smallest-sized catheter. A pyelogram with 5 per cent. sodium iodide showed no abnormality. Only 5 oz. of urine were obtained. A pyelostomy was performed on the left side by Lieutenant-Colonel N. L. Sheppard, R.A.M.C. The tissues were very oedematous. The left kidney was swollen and tense. The pelvis was blackish-blue in colour and was very distended. The ureter was hard and rubber-like. On opening the pelvis about 7 ml. of greenish-black mucoid material burst out under pressure. A brisk intrarenal haemorrhage occurred. A catheter was passed down the ureter from the pelvis to the bladder without obstruction. A tube was stitched into the pelvis of the kidney and was at once blocked by a blood clot; urine began to flow in 12 hours. The urine from the tube was blood-stained for two days; urine was passed by the urethra after 14 hours and was blood-stained for three days. 27.1.44. Blood urea 120 mgm. per 100 ml. Although the temperature fell it did not reach normal; the urine contained pus and a few red blood-corpuscles, and yielded a profuse growth of coliform organisms.

31.1.44. Blood urea 210 mgm. per 100 ml. Urinary urea 0.1 mgm. per 100 ml. Chlorides sub-normal.

1.2.44. A blood culture was sterile; total leucocytes 8,000 per c.mm., with polymorphonuclear leucocytes 68 per cent.

2.2.44. The patient feels well but continues to have fever; blood urea 70 mgm. per 100 ml. Oedema of right lumbar region.

3.2.44. Right kidney tender and enlarged, passing blood-stained urine from the right ureter.

17.2.44. Blood urea 45 mgm. per 100 ml.

27.2.44. An acute attack of renal colic on the right side, associated with haematuria, the temperature rising to 103° F. Pus cells disappeared from the urine on 3.3.44, and albumin nine days later. An intravenous pyelogram on 20.3.44 showed both kidneys functioning normally, although the right showed a slight degree of hydronephrosis.

The patient made an uneventful recovery, although the pyrexia only gradually subsided under alkalies. He returned to England 24.4.44.

The total protein in the cerebrospinal fluid was 80 mgm. per 100 ml. and cells 44 per c.mm. The crystals found in the urine and kidney pelvis were similar to those produced from sulphamerazine by acetylation.

As previously noted, no toxic results have been seen as a result of the use of mepacrine or quinine immediately after a sulphonamide.

THE EFFECT OF MUSCULAR EXERCISE IN A HOT MOIST ENVIRONMENT ON THE MEPACRINE CONCENTRATIONS OF BLOOD, PLASMA AND URINE

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The experiments described in this paper were carried out in 1944. They were designed to determine whether continued exertion in a hot environment would materially lower the plasma concentrations reached on a suppressive mepacrine dosage régime, and thus account for the occasional apparent failure of such régimes to suppress malaria. Under the conditions of the experiments it was found that the urinary excretion of mepacrine increased, but there was no corresponding reduction of plasma concentration.

EXPERIMENTAL

Twenty-two healthy volunteers were given 100 mgm. mepacrine daily for several weeks. They then worked for four hours in a hot chamber on four or eight consecutive days, depending on the experiment. Blood and plasma mepacrine concentrations and urinary mepacrine output were measured at standard times before, during and after the period in the hot chamber.

The Hot Chamber

This was a room 8 ft. by 7 ft. by 11 ft., with double walls of asbestos boarding and heated by thermostatically controlled electric radiators. The temperature and humidity, measured by a wet- and dry-bulb thermometer, were taken at half-hourly intervals. These were maintained at 32–35° C. and 70–85 per cent. respectively.

Muscular Exercise

The exercises were planned with the advice of Major J. Lovelock, R.A.M.C., and Professor C. G. Douglas, F.R.S.

The time-table for the four hours in the hot chamber was as follows :

10.00–10.30 hours.—Rest and acclimatization.

10.30–12.30 hours.—Periods of five minutes' exercise alternating with five minutes' rest.

12.30–13.00 hours.—Rest and lunch in chamber.

13.00–14.00 hours.—Periods of five minutes' exercise alternating with five minutes' rest.

During the five minutes' exercise the subject, while in the hot chamber, (a) climbed a stepladder (1.66 metres high), (b) pedalled on a bicycle ergometer against a resistance of 5 lb. at a rate of 59 pedal revolutions per minute (working at the rate of 736 kgm.-metres/min.) or (c) lifted a weight of 35 kgm. 25 times to a height of 0.46 metres.

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This programme had to be reduced in many cases, but the total calculated work done in four hours was always between 30,000 and 40,000 kgm.-metres. All the subjects were young and some were athletic, but most found this work a severe test of their physical endurance.

Experimental Conditions

Fluid, food and salt were given freely but were measured. The subject was weighed on entering and leaving the chamber, and the volume of urine passed was measured. From these data the volume of sweat loss was calculated; the respiratory loss has been ignored. For the four-hour period the calculated sweat loss was of the order 1,500–2,500 c.cm. It is interesting to note that in no case was there a spontaneous demand for salt. A few volunteers took salt at first because they were told it would be good for them, but they refused it later.

Clinical Observations

The mouth-temperature, pulse-rate, respiration-rate and blood-pressure were taken at the beginning and at the end of the period in the hot chamber, and again half an hour later. The temperature and pulse-rate were also taken half hourly throughout the period.

The mouth-temperature rose steadily to 101–102° F. throughout the four hours; work was stopped while the temperature was above 102° F. Half an hour after leaving the chamber it had usually fallen to 99° F. The pulse-rate rose to 120–150 per minute within a few minutes of starting exercise and stayed at about that level, with a drop to 110 during the half-hour lunch interval. Half an hour after the end of the period the pulse-rate was usually between 90 and 110. The blood-pressure (systolic and diastolic) remained at normal levels throughout the period.

Plan of Mepacrine Dosage and Estimation

(a) Four soldier volunteers were given 100 mgm. mepacrine daily at 09.30 hours for 29 days. They worked for four hours in the hot chamber on each of the 22nd–29th days. Minimal blood and plasma mepacrine concentrations* were measured (Masen, 1943) on the 1st–30th days, and then every second day till the 50th day. On the 1st, 7th, 14th, 19th, 22nd and 29th days estimations of blood and plasma mepacrine concentration were made at the 1st, 2nd, 4th and 8th hour after the drug had been given so as to obtain a post-absorption curve. The 24-hour urinary mepacrine excretion was measured on the same days as the minimal blood and plasma concentrations.

(b) Six undergraduates (Oxford) were given 100 mgm. mepacrine daily at 09.00 hours from the 1st to the 14th day and again from the 43rd to the 49th day. They exercised for four hours in the hot chamber on the 46th–49th days. Minimal blood and plasma concentrations and 24-hour urinary mepacrine excretion were measured on the 30th–43rd days and subsequently every second day till the 55th day. Blood and plasma mepacrine concentrations were also measured 1, 2, 4, 8, 10 and 12 hours after the drug had been given on the 36th, 39th and 42nd days.

* Minimal concentrations are defined as those concentrations reached on the dosage régime in question 24 hours after taking 100 mgm. (i.e., immediately prior to a dose).

(c) Eight undergraduates were given 100 mgm. mepacrine daily at 09.00 hours for 42 days. They exercised for four hours in the hot chamber from the 39th to the 42nd days. Minimal blood and plasma levels and 24-hour urinary mepacrine excretion were measured on the 30th-42nd days and subsequently every second day till the 55th day. Blood and plasma mepacrine concentrations were also measured 1, 2, 4, 8, 10 and 12 hours after the drug had been given on the 36th, 39th and 42nd days.

(d) The object of this experiment was to obtain plasma mepacrine concentrations uncomplicated by the active absorption of the drug, and to compare those on a hot-chamber day with those on a control day under normal conditions. To attain this four undergraduates were given 100 mgm. mepacrine daily at 09.30 hours for 22 days. Four hours were spent in the hot chamber on the 22nd and 23rd days. On the 23rd day blood and plasma mepacrine concentrations, urinary mepacrine excretion and white blood-cell counts were measured at 09.30 hours and 1, 2, 4, 8, 10, 12 and 24 hours later. The drug was resumed from the 24th day to the 31st day, and on the 32nd day a similar set of data was obtained.

RESULTS

Since the conditions of the experiments described in sections (a), (b) and (c) above were substantially the same, the results can be considered together.

The Minimal Blood and Plasma Levels

There was no change in the minimal blood and plasma levels which could be associated with exercise in the hot chamber.

The Form of the Post-Absorption Curve

The form of the post-absorption curve on the third day before exercise in the hot chamber was compared with that on the first hot-chamber day and with that on the last hot-chamber day in the 18 volunteers. The following were compared in each case: (i) the peak of the absorption curve; (ii) the time taken to reach the peak; (iii) the area of that part of the absorption curve between 0 and 8 hours above the minimal level.

There was no significant difference between the curves obtained in the three experiments.

The Urinary Mepacrine Excretion During the Period in the Hot Chamber

Each subject emptied his bladder on entering and leaving the hot chamber. The urinary mepacrine excretion was estimated for the four-hour period of exercise and for the remaining 20-hour period of the 24 hours. In each 24-hour period for each subject the difference—hourly mepacrine excretion during the four-hour period less hourly mepacrine excretion during the 20-hour period—was determined.

The results of experiment (d) were similar to those of the other experiments. There were no significant changes in plasma or whole-blood concentrations while the subjects were in the chamber or subsequently. There was again a significant increase in the hourly mepacrine output during the period spent in the hot chamber. No corresponding increase in output was observed in the period of eight hours immediately following release from the chamber (see table, section 2).

TABLE

Hourly mepacrine output during and after exercise in a hot moist chamber

	No. of differences	Mean differences	SD	SE of mean
1. Experiments (a), (b) and (c) Differences in individuals between amount of mepacrine excreted per hour while <i>in</i> the hot chamber and during the following 20-hour period	79	+ 0.16 mgm.	0.3	0.03
2. Experiment (d) i. Differences in individuals between amount of mepacrine excreted per hour while <i>in</i> the hot chamber and the corresponding period on the control day, i.e., 0-1, 1-2 and 2-4 hours ...	12	+ 0.31 mgm.	0.4	0.13
ii. Differences in individuals between amount of mepacrine excreted per hour <i>after leaving</i> the hot chamber and the corresponding period on the control day, i.e., 4-8, 8-10 and 10-12 hours	12	+ 0.01 mgm.	0.6	0.26

DISCUSSION

The increase in urinary mepacrine excretion observed while in the hot chamber can be explained by the observation that the urinary mepacrine excretion varies directly with the urinary ammonia excretion (Army Malaria Research Unit, 1944). A more acid urine, with a raised ammonia concentration, would be expected in men taking violent exercise. In general, the inference to be drawn from these experiments is that work in a hot atmosphere does not greatly modify the plasma mepacrine concentration reached on a suppressive dosage régime of 100 mgm. daily.

SUMMARY

1. Twenty-two volunteers were given 100 mgm. mepacrine daily for several weeks. Each then exercised strenuously for four hours a day for 4-8 days in a hot moist chamber.
2. There was no change in the minimal plasma or blood concentrations.
3. The urinary excretion of mepacrine was slightly increased during the period of exercising in the hot chamber.

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THE EXCRETION OF MEPACRINE IN THE FAECES

BY

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Results of experiments in which the faecal output of mepacrine was measured on the suppressive dosage régime of 100 mgm. daily are given below. In some individuals nearly 30 per cent. of the dose may be excreted in the faeces. In other subjects the faecal output is much smaller. In general, our experimental subjects could be divided into groups: those who excreted large amounts of the drug in the faeces and those who excreted small amounts. In view of the small output of mepacrine in bile (Army Malaria Research Unit, 1946), the faecal output after intramuscular injection of mepacrine was estimated in volunteers who had not previously had the drug orally. The relatively high faecal output found in these experiments indicated that mepacrine can be in some ways excreted into the gut. The faecal output is, therefore, not apparently directly related to non-absorption of the administered dose. In our experiments, the faecal output did not significantly alter over long periods of administration of mepacrine. No close relation was established between faecal output and whole-blood and plasma concentrations or urinary output.

EXPERIMENTAL

Mepacrine was estimated by the modified Masen's method described in previous communications (Masen, 1943).

The entire specimen of human faeces was ground in a large mortar, and small quantities of water were added until the final volume was 1 litre for a 24-hour sample and 3 litres for a three-day specimen. The suspension was well stirred before sampling, since it was found that, if it were allowed to settle, the mepacrine content of the supernatant fluid was less than that of the sediment. Analysis was performed on 1 ml. samples of the faecal suspension.

Mepacrine hydrochloride is quite stable in faeces over a period of days, even in daylight and at room-temperature. It was therefore possible in our balance experiments to collect both one- and three-day samples without fear of loss of mepacrine. The faeces passed during the three days were collected in large stone jars, which were kept closed and left at room-temperature. At the end of the three-day period suspensions were made as described above.

The validity of the methods used in these experiments is discussed in an appendix to this paper.

RESULTS

Random Sampling

Random three-day sampling was carried out in volunteers who had been taking 100 mgm. mepacrine hydrochloride daily for periods varying from three days to six

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months. The results are set out in Table I. It will be noted that at any one period individual differences in output were very great. The differences in output between those subjects who had received the drug for a similar length of time were also much greater than the differences between the means of each period. Analysis showed that these differences between the means were not significant.

TABLE I

Mepacrine in random samples of faeces (expressed in mgm./24 hours) measured on three-day specimens from subjects receiving 0.1 gm. mepacrine hydrochloride daily

After 3 days	After 1 week	After 3 weeks	After 6 weeks	After 3 months	After 6 months
7.5	9.5	16.0	1.8	4.1	12.2
15.1	20.5	15.0	3.6	12.3	7.6
0.3	18.1	4.2	2.4	17.4	9.1
17.7	33.2	3.2	17.6	11.2	
11.1	10.2	1.1	18.9	9.6	
19.6		15.4	16.3	0*	
		33.7		20.8	
		27.5		38.2	
				15.7	
				19.2	
				18.3	
				31.3	
				15.0	
Mean 11.9	18.3	14.5	10.1	15.8	

* Subject passed no faeces for three days.

Repeated Estimation of Faecal Output in the Same Subjects

An attempt was made to eliminate the great variation in output between subjects. Six volunteers were given 100 mgm. mepacrine hydrochloride daily for 13 weeks, and during the 1st, 6th and 13th weeks two three-day samples of faeces were collected for estimation. The concentrations of mepacrine in the blood and plasma and the total amount eliminated in the urine were also determined during the three-day periods. The results are given in Tables II and III.

TABLE II

Excretion of mepacrine in faeces (mgm./day) of volunteers receiving 0.1 gm. mepacrine hydrochloride daily; each figure the mean of two three-day periods

Volunteer no.	1st week	6th week	13th week	Total
267 } Group A {	0.3	1.8	10.4	12.5
264 } {	7.5	3.0	14.3	24.8
265 } {	15.1	3.4	8.7	27.2
271 } Group B {	10.3	26.3	18.7	55.3
269 } {	17.7	16.5	26.9	61.1
274 } {	20.0	21.9	23.1	65.0
Total ...	70.9	72.9	102.1	245.9

TABLE III

Mepacrine in blood, plasma and urine of volunteers receiving 0.1 gm. mepacrine hydrochloride daily; each figure the mean of six daily estimations

Volunteer no.	Plasma mepacrine ($\mu\text{gm./l}$)			Whole-blood mepacrine ($\mu\text{gm./l}$)		Urine mepacrine (mgm./day)		
	1st week	6th week	13th week	1st week	13th week	1st week	6th week	13th week
267	17	20	25	46	132	0.7	2.8	3.4
264	7	24	28	19	114	1.5	3.9	6.9
265	7	27	20	42	108	0.6	2.8	3.1
271	8	22	27	32	135	1.2	3.0	3.7
269	7	22	18	22	108	0.5	1.2	2.5
274	8	18	27	35	113	0.5	2.2	3.9
Mean ...	9	22	24	33	118	0.8	2.7	3.9

Examination of the results shows that during the 6th week the faecal excretion of mepacrine was no greater than in the 1st week, but there was an apparent slight increase in excretion in the 13th week. This increase is not, however, significant ($t = 2.04$, which corresponds to $P = 0.1$; for $P = 0.05$, $t = 2.57$).

The results were subjected to an analysis of variance. This showed that there is a significant difference between the inter-row (individual) and residual variances ($z = 0.8613$; 5.0 per cent. = 0.6009), but no significant difference between the inter-column (time) and residual variances ($z = 0.278$; 5.0 per cent. = 0.7058). This means that the subject-to-subject variation in the daily amount of mepacrine excreted in the faeces (at any given time after the beginning of mepacrine administration) is greater than the variation in mepacrine output with time, observed in the same subject. As will be seen, this observation is important from the point of view of correlating changes in faecal output with blood plasma and urine mepacrine concentrations.

For convenience, the results of faecal determination in the six volunteers have been divided into two groups, A and B (Table II). Subjects in group A had a low mepacrine faecal output; those in group B had a higher output. Comparison of the blood and plasma mepacrine concentrations, or the daily urinary output in the six volunteers (Table III), shows, however, no similar differences within these two groups. It is evident that the total excretion of mepacrine in the faeces when the subjects are taking 100 mgm. daily is not very closely related to the excretion of the drug in the urine or to the concentrations reached in either whole blood or plasma. Nor is it intimately connected with daily variations in absorption of mepacrine from the gut, since, if changes in blood and plasma concentrations and urinary output of mepacrine are to be explained by differences in absorption, one would expect greater variation of these quantities in a group of subjects at any particular time than there would be in any given subject throughout the period of dosage. As will be seen from Table III, wide ranges of variation of the order of those seen in faecal output do not, in fact, occur in blood, plasma and urinary output.

Excretion of Mepacrine in Faeces after Intramuscular Injection

Table IV gives the mepacrine content of the faeces of two subjects who had received

mepacrine by intramuscular injection. Mepacrine was excreted in these subjects, although no drug was given orally. Thus the presence of mepacrine in the faeces does not necessarily mean that the drug has not been absorbed. Excretion into the gut after absorption may occur even after oral administration.

TABLE IV
Elimination of mepacrine in faeces after intramuscular injection of the drug

Volunteer no.	Dose of mepacrine musonate given by intramuscular injection (gm.)	Mepacrine content of faeces (mgm.)	
		1st day after injection	2nd day after injection
274	0.6	13.8	9.4
263	0.9	15.2	6.1

Mepacrine Excretion in the Faeces of Rats

It was suggested at one time that the total excretion of mepacrine might, after a long period of daily administration of the drug, rise sufficiently to influence the plasma concentration and lower it to the point at which suppression of malaria might be inadequate.

TABLE V
Mepacrine in faeces of rats receiving 5 mgm./kgm. mepacrine hydrochloride daily; measured on a three-day specimen and recorded as mgm./24 hours

Day	Rat A	Rat B	Rat C
1	0.03	0.04	0.32
4	0.29	0.26	0.36
7	0.21	0.19	0.27
10	0.27	0.19	0.25
13	0.20	0.27	0.27
16	0.20	0.20	0.21
19	0.13	0.15	0.15
22	0.14	0.20	0.21
25	0.16	0.15	0.19
28	0.16	0.15	0.16
31	0.11	0.11	0.14
34	0.12	0.10	0.14
37	0.14	0.17	0.20
40	0.13	0.10	0.12
43	0.10	0.11	0.11
46	0.11	0.11	0.17
49	0.18	0.14	0.16
52	0.13	0.09	0.11
55	0.13	0.14	0.21
58	0.19	0.16	0.16
61	0.15	0.17	0.15
64	0.11	0.11	0.20
67	0.16	0.18	0.21
70	0.17	0.19	0.19
73	0.19	0.19	0.25
76	0.18	0.15	0.19
79	0.12	0.13	0.15
82	0.13	0.12	0.14
85	0.18	0.19	0.16
88	0.20	0.18	0.21

Our experiments show that such an increase in output did not occur in a group of subjects watched over a period of 13 weeks ; nor was it indicated in samples taken from the subjects at random after 3-6 months on the suppressive course. We were unfortunately unable to carry our human experiments further than six months, but we repeated the work in rats, following the faecal excretion of mepacrine in three animals for a period of 12 weeks—a very considerable time in the life of a rat. The animals were given mepacrine (5 mgm. per kgm.) by stomach-tube daily, and estimations were made on three-day samples. As will be seen from Table V, there was no significant increase in faecal mepacrine output, once equilibrium between input and output had become established, over the period of the experiment. This finding is in agreement with the human results. There is thus no evidence that faecal excretion of mepacrine increases after long periods of dosage.

APPENDIX

Methods of Extraction of Mepacrine from Faeces

At the time when these experiments were being carried out, two methods of estimating the mepacrine content of faeces were in use in laboratories in England. The method (the emulsion method) adopted by this Unit is described above. The other consisted in grinding the sample of faeces with anhydrous sodium sulphate, taking up the resultant powder in distilled water, shaking and standing until insoluble material settled, and estimating the drug content of the supernatant fluid (Reid, 1945). In our hands the two methods gave completely incompatible results, so that it became necessary to check them both carefully before embarking on big-scale experiments. We finally adopted the emulsion method, as we found that large amounts of mepacrine were removed from solution in the second method as the result of adsorption on to solid particles. If, however, instead of estimating only the mepacrine content of the supernatant, the content of the whole sample of faeces in sodium sulphate was measured, the results were comparable with those obtained by the emulsion method.

The figures in Table VI show the percentage recoveries of mepacrine from mixtures of faeces and known amounts of mepacrine, treated by total extraction of an emulsified sample and a sample rubbed up with sodium sulphate.

TABLE VI

Recoveries of mepacrine from faecal emulsions and from faeces ground with sodium sulphate

Faeces sample	Method	Percentage recovery	
		Immediately on mixing	After 3 days
A	1	98.5	93.5
	2	80	82.5
B	1	96	101
	2	97.5	92
C	1	122	107
	2	109	71
D	1	107	112
	2	105	83

Method 1: Total extraction of sample (faecal emulsion) by Masen's method.

Method 2: Total extraction of sample (faeces ground with sodium sulphate) by Masen's method.

A comparison of the emulsion method and Reid's method is illustrated in Table VII. It will be seen that the latter method gives consistently low results. That these results were due to adsorption of the drug on the solid particles in the faeces was shown by the recovery of equivalent amounts of mepacrine from the deposit.

TABLE VII

Recovery of mepacrine from faeces and mepacrine mixture : comparing two methods

Faeces	Percentage recovery (after mixing three days or longer)		Time after mixing faeces and mepacrine
	Total extraction of faeces ground with sodium sulphate	Extraction of supernatant after mixing faeces ground with sodium sulphate with water	
A (solid)	91	59	28 days
*A "	80	38	32 "
B "	85	37	17 "
*B "	70	30	24 "
F "	76	32	3 "
G (diarrhoea liquid)	90	78	3 "
†Silica	5	7.5	15 minutes

* Checked by Dr. Gage in I.C.I. (Dyestuffs) Laboratories.

† Faeces containing known amount of mepacrine rubbed up with sand and extracted immediately after mixing.

TABLE VIII

Stability of mepacrine in faecal suspensions

A. Faeces from subjects taking 0.1 gm. mepacrine hydrochloride by mouth				
Mepacrine expressed as gm. output per 24 hours				
	Subjects	Day 0	Day 1	Day 3
Daylight and room-temperature ...	256	2.6	3.2	3.0
	244	5.5	5.7	5.2
	249	6.4	5.8	6.6
	284	7.7	6.5	6.4
Dark and refrigerator	256	2.6	3.1	3.1
	244	5.5	4.8	5.1
	249	6.4	7.2	6.7
	284	7.7	7.0	6.6

B. Mepacrine 500 μ gm. added to 100 gm. human faeces

Mepacrine concentrations in mgm. per 100 gm. faeces

	Subjects	Day 0	Day 1	Day 3
Daylight and room-temperature ...	A	0.56	—	0.48
	B	0.54	—	0.50
	C	0.51	—	0.52
Dark and room-temperature	A	0.56	—	0.54
	B	0.54	—	0.50
	C	0.57	—	0.57
Dark and refrigerator	A	0.56	—	0.50
	B	0.54	—	0.48
	C	0.51	—	0.58

Stability of Mepacrine in Faeces

Once the method of extraction of faeces had been decided, experiments were carried out to discover whether mepacrine was easily destroyed by faeces. It was found that mepacrine in faeces was remarkably stable.

Table VIII, A, shows the mepacrine content of samples of the faeces of four volunteers who were receiving the drug by mouth. The faeces in these cases were emulsified as described above (emulsion method) and kept under various conditions for three days. Table VIII, B, shows the recoveries of as little as 0.5 mgm. mepacrine added to faeces

TABLE IX

A. Stability of mepacrine in faeces

A. Faeces stored as suspensions

B. Faeces ground with sodium sulphate

- (1) 1.25 mgm. mepacrine hydrochloride added to 100 gm. *human faeces*
 (2) 0.200 " " " " " "
 (3) 0.100 " " " " " "
 (4) Faeces from patient taking 0.1 gm. mepacrine hydrochloride daily

		Mepacrine recovered, in mgm. (total extraction of sample)		
		Immediate extraction	After 24 hours	After 72 hours
(1) Experiment I	A ...	1.20	1.23	1.26
	B ...	1.22	1.22	1.15
Experiment II				
	A ...	1.23	1.14	1.17
	B ...	1.00	1.11	1.03
(2)	A ...	0.215	0.216	0.226
	B ...	0.211	0.179	0.167
(3)	A ...	0.122	0.120	0.108
	B ...	0.109	0.086	0.071
(4)	A ...	1.29	1.30	1.44
	B ...	1.30	1.20	1.32

B. Percentage recovery of mepacrine added to faeces (faeces ground with sodium sulphate)

- i. By total extraction by Masen's method
 ii. By Reid's method

	Method	Percentage recovery				
		Immediately on mixing	Hours after mixing			
			3	24	48	72
a. Solid faeces F ...	<i>i</i>	91	91	91	80	76
	<i>ii</i>	—	56	39	39	32
b. Liquid faeces G ...	<i>i</i>	80	80	88	86	90
	<i>ii</i>	—	85	80	74	78

and treated similarly. In both cases there was no significant change of concentration after three days, the variations seen in the table being no greater than the variations in replicate determinations on a single suspension.

Other experiments in which small amounts of mepacrine were added to faeces and extracted at various times after mixing confirmed these results (Table IX, A). It was found that, provided extraction was complete, good recovery of mepacrine was possible more than a month after mixing the faeces with sodium sulphate and storing at room-temperature (see Table VII). (Simple emulsions could not be kept for this length of time.) Measurement of the supernatant mepacrine content only, however, produced results which varied with the specimen of faeces; for example, recoveries by this method from solid faeces were low, whereas recoveries from liquid faeces were good (see Table IX, B). This apparent loss of mepacrine was due to adsorption of the drug on to solid particles in the faeces, and not to its destruction.

SUMMARY

1. Six subjects were given 100 mgm. mepacrine hydrochloride daily for 13 weeks. The mepacrine content of three-day samples of faeces was determined during the 1st, 6th and 13th weeks. The mepacrine output in the faeces did not increase significantly over this period.
2. Three-day specimens of faeces were collected at random in a group of subjects who had taken 100 mgm. mepacrine hydrochloride daily for periods varying from one week to six months. The output of mepacrine at six months was not significantly higher than after shorter periods on the drug.
3. The mepacrine content of three-day samples of faeces was estimated every three days in three rats receiving 5 mgm. mepacrine per kgm. for 12 weeks. There was no increase in mepacrine output over this period.
4. No close relation was found between the output of mepacrine in faeces and the output in the urine or the concentration in whole blood or plasma.
5. Mepacrine was found in the faeces of subjects who received the drug intravenously.
6. Mepacrine hydrochloride was found to be stable for at least three days in faecal suspensions at room-temperature.

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THE RÔLE OF *ANOPHELES GAMBIAE* VAR. *MELAS** IN THE TRANSMISSION OF MALARIA IN THE VICINITY OF FREETOWN ESTUARY, SIERRA LEONE, 1943

BY

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INTRODUCTION

Freetown has been the site of investigation of the problem of malaria by a line of research workers since 1900, when mosquito-surveys were conducted by Ross and Christophers. Between 1920 and 1940 Blacklock, Gordon, Macdonald, Davey and others associated with the Sir Alfred Lewis Jones Research Laboratory, a field station of the Liverpool School of Tropical Medicine, carried out investigations into the prevalence of malaria and of anopheline mosquitoes in Freetown and its immediate vicinity, with resultant recommendations for control of the disease; the extent to which such recommendations were put into effect by the local Government was in proportion to the funds made available.

The 'invasion' of this important strategical port in 1940 by thousands of personnel of the three fighting Services, with the rapid construction of camps along the southern shore, was followed by heavy malaria incidence among those personnel. The increasing use of the estuary as an anchorage for shipping of all types, including troop transports, drew attention to the potentialities of infection of mercantile marine personnel and troops in transit by the flight of infected mosquitoes to shipping lying off the shore.

In 1940-41 Professor Blacklock with Dr. Carmichael Wilson conducted a survey in relation to malaria contracted in the shipping anchorage, and their report formed the basis for subsequent control-measures, undertaken jointly by the civil and military medical departments; field control was directed against the fresh-water breeding-places of *Anopheles gambiae* between Aberdeen and Kissy.

In September, 1941, an Army Malaria Field Laboratory arrived in Freetown; Major C. R. Ribbands, R.A.M.C., who joined this unit, had been studying brackish-water breeding of *A. gambiae* on the shore of Aberdeen Creek for some months, and was convinced that this melanic form of *A. gambiae* was a vector of malaria on Aberdeen peninsula; its breeding-places were loosely connected with the mangrove area and were uncontrolled.

In July, 1942, the Colonial Office Research Entomologist, Dr. Muirhead Thomson, arrived in the Colony for the specific purpose of investigation into the ecology of *A. gambiae*. Within a few months he had observed and established the differential characteristics between the eggs of the salt-water melanic form and the fresh-water type-form of *A. gambiae*.

This important observation enabled the Army laboratory, with the advice and active collaboration of Muirhead Thomson, to conduct in 1943 the investigation which may

* At the time when this paper was written the specificity of *A. melas* had not been confirmed.

be summarized in the question 'Is *A. gambiae* var. *melas* an important vector of malaria in the vicinity of the shores of Freetown estuary?' The results of this investigation and the control-measures adopted are the subject of this paper.

PRELIMINARY APPRAISAL OF THE MALARIA SITUATION

THE FREETOWN ESTUARY

(Appendix I, map 1)

The estuary is 7-8 miles wide at the outlet and extends into the interior some 9-10 miles; the narrowest part lies opposite Cline Town and Kissy and is between three and four miles wide; the eastern end divides into two channels, a wide northern channel traversable by cargo-vessels to the port of Pepel, and a narrow southern one which is the outlet of the Bunce River. Dense mangrove forest lines the north-east, east and south-east borders of the estuary, extending along the southern shore in a westerly direction as far as Wellington and in an easterly direction to the upper reaches of the tidal Bunce River. A characteristic of the estuary is the variation in the tide-levels, spring tides reaching a height of 11 feet, an important factor in relation to breeding-areas of *A. gambiae* var. *melas*.

The Southern Shore. This is the northern fringe of the mountainous peninsula forming the Colony of Sierra Leone, and is rock-bound from Aberdeen peninsula in the west as far as Wellington in the east, a distance of nine miles. Between Aberdeen peninsula and Freetown is a mangrove-lined creek two miles in length, a spur of high ground being interposed between its eastern edge and the suburbs of the town. Freetown itself lies at the foot of encircling hills, which rise abruptly to a height of 900 feet. The fairly level coastal belt extending to the east varies in width from half a mile to two and a half miles until Wellington is reached. Here the mangrove belt commences and attains in places the width of a mile; the coastal plain widens, and at Jui and Hastings two low-lying peninsulas jut out into the mangrove of the Bunce River creek. Continuing to the east the mountains cease at the low-lying neck of country which links the Sierra Leone peninsula to the mainland; numerous tidal creeks, offshoots of the Bunce River, invade this area; on one of these creeks lies the town of Waterloo, 16 miles from Freetown.

The Northern Shore. In distinction to the southern shore, the northern shore is low-lying and devoid of hills; long sandy elevations, at the back of which lie fresh- or brackish-water marshes, are typical of the seven miles of shore nearest to Freetown; in the wide northern extension of the estuary are situated several islands containing similar marshes.

The Eastern Shore. Dense mangrove forest, in places several miles in depth, obliterates the shore-line; the absence of hills is again in contrast to the mountainous southern shore.

The Shipping Anchorage. The anchorage, under war conditions, covers a large area, as indicated on the map, but the main berths lie nearer to the southern shore off Aberdeen, Freetown and Kissy. Certain cargo-vessels proceed up the north-eastern reaches of the estuary to Pepel, and may anchor overnight en route.

Service Installations. Naval and military installations lie along the southern shore between Aberdeen and Wellington; airfields and seaplane-bases are sited in relation to the mangrove-lined creeks of the eastern end of the estuary.

THE RESERVOIR OF INFECTION

The normal African population along the southern shore has been augmented during the war by influx from the Protectorate, so that accurate figures are impossible to obtain. The urban area of Freetown and its vicinity has a population of between 50,000 and 70,000; the 10 villages between Aberdeen and Waterloo and the intervening hamlets house many thousands of African children.

The estimation of the spleen-rate and parasite-index of sections of the African population has been carried out at intervals since the report of Macdonald in 1926. Peaston and Renner (1939) in 1935 ascertained the 'splenic index' in school-children of from three to 10 years of age, in relation to the completion of a drainage scheme. In 1940 Blacklock examined the blood of children (approximately 200) in Kissy on the southern shore and in various villages along the northern shore, and considered that 100 per cent. would show parasites in the circulation if examinations were frequent enough.

The following table summarizes the situation.

Investigator and year of investigation	Place		Age-group	No. examined	Spleen-rate %	Parasite-index %
Macdonald, 1926	Freetown :					
	Hyperendemic area		0-10	265	71	77
	Endemic area		0-10	586	49	42
Peaston and Renner, 1935	Freetown :					
	Hyperendemic area		3-10	486	43	38.5
	Endemic area		3-10	465	44	34
Army Malaria Field Laboratory, 1943	Aberdeen	Dry season	5-10	38	55	34
		Wet "	5-10	46	63	30
	* Cline Town	Dry "	5-10	50	32	22
		Wet "	5-10	45	51	0
	* Kissy	Dry "	5-10	172	35	24
		Wet "	5-10	110	49	9
	* Murraytown	Dry "	5-10	57	51	21
		Wet "	5-10	53	49	8
	* Wellington	Dry "	5-10	50	54	36
		Wet "	5-10	61	44	11.5
	Hastings	Dry "	5-10	109	59	11
		Wet "	5-10	116	45	34
	Waterloo	Dry "	5-10	166	44	Not done
		Wet "	5-10	178	43	48
	Villages : east Waterloo	Dry "	2-10	135	76	32
		Wet "	2-10	100	53	38
	Villages : north shore	Dry "	2-10	834	49	Not done
		" "	2-10	52	Not done	42

* 'Mepacrinized' school-children; note decrease in parasite-index. *Plasmodium falciparum* is the usual species of parasite; mixed infections with *Plasmodium malariae* are occasional; *Plasmodium vivax* is a rarity.

An extensive reservoir of infection obviously exists throughout the territory around the Freetown estuary, the small African villages being a larger source of infection than

the towns, such as Waterloo and Freetown. The children are the gametocyte-carriers, and on the average the younger the child the more frequent is the presence of gametocytes in the peripheral circulation. Immunity is acquired rapidly in the rural areas, so that clinical malaria is uncommon in older children and adults; in Freetown urban area control-measures have reduced the density of mosquito production, so that the attainment of maximum immunity is delayed several years as compared to the rural village; indeed, clinical malaria in the better-housed African adult of Freetown is increasing, which tends to produce the mistaken view among the educated Africans that health-conditions in relation to malaria are becoming worse despite the control-measures adopted.

THE VECTOR MOSQUITOES

The mosquito-population has been studied and reported on by the research workers of the Sir Alfred Jones Laboratory during the period 1920-40; the sites of these investigations, however, appear to have been limited to the urban area of Freetown from the King Tom peninsula in the west to Cline Town in the east, Kissy village, and certain areas in the Protectorate such as Daru and Makeni. In 1932 Gordon and others quoted *A. costalis* and *A. funestus* as the only species of *Anopheles* of importance in the transmission of malaria in Freetown and Kissy. In later years attention has been drawn to the presence of other vectors in the Colony, namely, *A. hancocki*, *A. nili* and *A. hargreavsi*, all of which appeared to have a localized distribution.

The melanic coastal form of *A. gambiae*, known as *A. gambiae* var. *melas*, has not been described as of general occurrence by the research workers above referred to. Gordon, however, found occasional specimens in Freetown, but never on the scale of density of production described by Barber and Olinger (1931) as originating from brackish coastal waters near Lagos, Nigeria. Evans (1938) states that the melanic coastal form is widely distributed in West Africa from Gambia to Nigeria.

During 1940 Blacklock and Wilson (1941b) found that one-fifth of the mosquitoes caught in houses on the northern shore were *A. gambiae* var. *melas*, the differential character employed being the 4-banded palp. Larval catches from various fresh- and brackish-water breeding-places were bred out with a resultant proportion of var. *melas*. These authors concluded: 'It is evident that a study of the bionomics of brackish water breeding *A. gambiae* and *A. gambiae* var. *melas* in the estuary region may result in useful indications for special means of prevention.' They had found four mosquitoes infected in the salivary glands out of 74 dissections of *A. gambiae* var. *melas*. Lastly, they demonstrated by collection on ships that culicine and anopheline mosquitoes reached them in fair numbers and, on occasions with favourable winds, in large numbers; by dissection they found two infected anophelines.

MALARIA CASUALTIES UNDER WAR CONDITIONS

Thousands of personnel of all Services were quartered in various parts of the Colony; advantage was taken of the less malarious areas on the hills, but of necessity a large proportion of personnel, particularly of the Royal Navy and the Royal Air Force, were accommodated along the southern shore of the estuary from Aberdeen to Waterloo; operational requirements resulted in R.A.F. installations being sited, in relation to the south-easterly reaches, in places which in the wet season were intensely malarious. War transports, conveying many thousands of Servicemen to other theatres of war, were

berthed in the anchorage for several days at a time, though personnel were not permitted ashore. The northern shore was not occupied by the Services.

Under conditions of war malaria statistics are of little value to the military malariologist, except as a gross estimate of the general situation in a particular territory. For individual camp-sites, airfields, zones or districts the total of cases is not always available, and total numbers of personnel remain secret for Service reasons; no account is taken of fluctuations in numbers of personnel from week to week or in changes of location of individuals; the incidence of malaria is vitally affected by variations in the efficiency of antimalaria discipline regarding personal precautions in the proper use of nets, protective clothing, etc.; related also to these factors are the presence or absence of 'metal-gauze screening' to quarters, and the influence on the individual of the regulation dose of the suppressive drug, if taken.

Statistics of the incidence of malaria are not given in this paper; it may be said that all measures of control applied have caused a pronounced decrease in the incidence among Servicemen; as an example, on a particular airfield adjoining the estuary practically all the personnel had three or four attacks of malaria during one year's tour of service in 1940-41, whereas in 1943 the incidence averaged out to half the personnel escaping an attack of clinical malaria and the remainder each having one attack involving hospitalization.

Troopships, which had lain in the anchorage a few days in the season of intense mosquito-breeding, produced cases of malaria before reaching Cape Town, the next port-of-call; it must be noted that the number of cases was small in proportion to the numbers of personnel aboard, but the contraction of malaria out on the waters of the estuary was proven.

CONTROL-MEASURES

In Freetown urban area and its immediate vicinity control-measures have been directed at the breeding-places of anopheline vectors, mainly *A. gambiae*; in the dry season these are the rocky-bedded hill streams, canalization being the 'permanent' method of control where it is considered to be a practical and economic proposition; in the wet season sunlit pool and seepage breeding over the whole area replaces breeding in streams, which have become torrents; in the urban area 'permanent' control has resolved itself into adequate surface-drainage. 'Permanent' measures have been steadily advanced during the last 15 years by the Government Medical and Public Works Departments, the limiting factor being finance. Under war conditions the sanitary staff was augmented, and 'temporary' control-measures were extended beyond the urban limits, principally in the Kissy direction; routine adult-mosquito destruction with pyrethrum spray in dwellings, drainage and 'oiling' of breeding-places, later supplemented by the use of Paris green, became generalized.

Estuary village malaria was uncontrolled until the commencement of the war. As a result of the report of Blacklock and Wilson (1941b), adult-mosquito destruction was undertaken in 1941 by the civil medical department in certain villages on the northern shore as a protective measure for shipping. 'Temporary' field control-measures, above referred to, were instituted by military medical services in relation to such installations as camp-sites, airfields, etc.; these measures were supplementary to the intra-unit protective personal precautions of the use of mosquito-nets, adequate clothing, repellents and

the destruction of adult mosquitoes; metal-gauze screening of quarters, etc., and the regular taking of 'suppressive' mepacrine by the individual were introduced.

The field control-measures continued to be directed against the predominant vector, *A. gambiae* type, a fresh-water breeder. The foresight of Major C. R. Ribbands in establishing in 1940 permanent mosquito-catching stations—a system which was gradually extended—brought to notice the fact that in certain areas a large proportion of the mosquito-population was the melanic coastal form of *A. gambiae* as described by Barber and Olinger (1931) in Nigeria; no existing methods of control touched possible brackish-water breeding-places. Similarly, in 1941 Blacklock and Wilson described the presence of this melanic form in the estuary hamlets on the northern shore.

It was evident that a blank of unknown dimension existed in entomological data and consequently in malaria-control measures also.

THE RÔLE OF *ANOPHELES GAMBIAE* VAR. *MELAS* IN MALARIA TRANSMISSION

From the point of view of the malariologist, who under war conditions must implement sound and, if possible, permanent control-measures in a relatively short space of time, the following information was essential:

1. A simple method of differentiation of *A. gambiae* var. *melas* from *A. gambiae* type.
2. The breeding-places of *A. gambiae* var. *melas* in distinction to those of *A. gambiae* type.
3. The proportion of adult female *A. gambiae* var. *melas* infected with malaria.
4. The density of the *A. gambiae* var. *melas* population in relation to (a) *A. gambiae* type population, (b) 'other' vector anopheline population, (c) areas to be protected.
5. A permanent method of control aimed at the elimination of the breeding-places of *A. gambiae* var. *melas* if paragraphs 3 and 4 above justified the undertaking.

(For descriptive purposes in this paper the salt-water breeder *A. gambiae* var. *melas* is referred to as '*melas*,' the fresh-water breeder *A. gambiae* as 'type,' and the term '*A. gambiae*' includes both varieties.)

DIFFERENTIATION OF *melas* FROM 'TYPE'

Anopheles (myzomyia) gambiae Giles has been studied by many research workers since 1900; existing information was summarized by Evans in 1938. For the purposes of this paper the following information is relevant.

The Adult Female. Melanic coastal forms (var. *melas* Theobald) were described by Evans (1938) from material obtained by Barber and Olinger from brackish-water breeding-places near Lagos, Nigeria, in 1931. Apart from the general darker appearance of the thorax and abdomen and the black scaling on the wings, 25 per cent. showed 4-banded palps, 'an extra dark band of variable width being present in what would otherwise be the long apical pale band.' Barber and Olinger's (1931) work indicated that the distinctions of coloration were unstable under experimental conditions.

Ribbands, of an Army malaria laboratory, after 18 months' observations in Sierra Leone, reported in 1942 as follows: no satisfactory morphological distinction between 'type' and *melas* had been found; normal 'type' specimens were lighter in colour than *melas*, but some were as dark; the extra dark band on the palp of *melas* bore no relation

to the degree of salinity of the breeding-place; among the specimens of *melas* with the extra dark band the quantity of extra dark pigment varied continuously within very wide limits. (These observations were based on 'salinity' experiments aimed at determining a physiological distinction between *melas* and 'type' larvae.) A diagram of the 4-banded palp is given in Appendix I, diagram 1.

Robertson (1944), of an Army malaria laboratory, in the course of routine study in the Gold Coast and Sierra Leone from 1942 has not been able to demonstrate any obvious distinction between the terminalia of *melas* and of 'type.'

The Egg. In a brackish-water *A. gambiae* breeding-area at Aberdeen, Muirhead Thomson (1944), during the period July to December, 1942, observed a periodic appearance of large numbers of anopheline eggs. In the examination of these and in comparison with the 'type' egg, which had been adequately described in the literature, Thomson defined the following difference: in *A. gambiae* type (fresh water) the frills of the egg are quite separate from the floats on either side, whereas in *A. gambiae* var. *melas* (brackish water) eggs the frills almost touch the float. The binocular dissecting microscope readily reveals this differential feature, so that the dorsal view of a *melas* egg is recognizable by the trained laboratory assistant; associated with this difference is the broadness of the 'platform' centrally in *melas*, as compared to a constriction or narrowing in 'type' (Appendix I, diagram 2).

Further, Thomson demonstrated that the eggs laid by 4-banded *A. gambiae* were of the *melas* variety, and in consequence he concluded that female *A. gambiae* mosquitoes exhibiting 4-banded palps are, in fact, *A. gambiae* var. *melas*.

The Larva. Evans in 1931 reported nothing distinctive in the brackish-water larvae collected by Barber and Olinger in Nigeria. They found larvae in water with 46 per cent. sea-water, but noted that inland fresh-water-produced larvae did not survive in saline water in which melanic larvae came to maturity. Ribbands confirmed this observation in 1942, and as a result of experiment suggested the physiological test of exposure of larvae to 60 per cent. sea-water as a practical method of distinguishing 'type' from *melas*. Ribbands (1942) also described a constant differential characteristic between *A. gambiae* fresh-water and brackish-water larvae: the pecten of 'type' has about 4-5 long, slender teeth without spicules usually alternating with three groups of 3-6 short teeth with spicules. The ratio of length of short to ventral long tooth is about 2/5 to 3/4. *Melas* has no markedly longer teeth (ratio 5/6), and all the teeth are spiculate.

The Pupa. No differential characters have been described.

Conclusion. For the purposes of this investigation the differentiation of adult *A. gambiae* has been necessary, so that the 4-banded palp and the egg difference have been utilized and the larval differences have not been required.

THE BREEDING-PLACES OF *A. gambiae* var. *melas*

Evans (1938) summarized past records by saying that the melanic coastal form of *A. gambiae* is characteristically a brackish-water breeder. Blacklock and Wilson (1941a) described 'a widespread prevalence of *melas*' on the northern shore, and found a breeding-place at the outlet of a Freetown stream, 'in the reach affected by the tide.' Muirhead Thomson in 1942, after observation of egg-laying in fresh-water pools just above high spring-tide limit and within a few yards of *melas*-producing tidal pools, concluded that in the rain-season at least *melas* makes use of fresh water only to an almost negligible

extent. Early in 1942 Ribbands had shown, under experimental conditions, that the melanic form could reach full development in fresh- or up to 100 per cent. sea-water.

It is evident that the *melas* female is attracted to and lays her eggs in saline waters, and that dilution of saline water by the entrance of fresh water from adjacent seepages or by varying degrees of rainfall does not cause such water to become unattractive to the female; nor does the dilution adversely affect full development of the larvae. During 1942 and 1943 the research work of Muirhead Thomson (1944) revealed evidence that the *melas* female is not indiscriminate in her selection of saline pools, but has a preference for pools in a rich-looking, peaty, black soil associated with a particular variety of mangrove swamp and with the belt of sea-grass (*Paspalum*) frequently present at the high spring-tide level. The recognition of this type of mangrove, known as *Avicennia*, is simple; this is described in Appendix VI, B, together with its relationship to the common *Rhizophora* mangrove and to the sea-grass belt referred to above; cross-section diagrams in Appendix VI, A, illustrate these relationships to the *melas* breeding-zones, and photographs in Appendix VI, C, show the distinctive features of these tidal swamps.

Within the limits of the sea-grass belt and the *Avicennia* mangrove zone, the formation of the saline pools in the dry season is dependent on the tidal inflow reaching the zone, which normally occurs with the average spring tides; when the fortnightly spring tides fail to cover the zone, pools may be absent from a section of the area or from the whole area for weeks at a stretch. In the wet season consistent rainfall and the entrance of seepage water, together with the occurrence of the higher spring tides, maintain a condition of bog through the *Avicennia* mangrove zone and of 'pooling' in the sea-grass belt. Muirhead Thomson found that the *melas* female showed a preference for egg-laying in the sea-grass belt or the adjacent parts of the *Avicennia* mangrove zone; eggs or young larvae would be washed out by subsequent tides into the depths of the *Avicennia* marsh and there complete development. In the instances where a *melas*-producing sea-grass belt adjoined *Rhizophora* mangrove Thomson was not able to find larvae in the mangrove depths, though maturity was attained in the pools persisting in the sea-grass belt. In consequence of the conditions described and the ease of availability of blood-meals, a rapid increase of *melas* production occurs at the onset of the rains, reaching a peak of intensity within a few weeks; the village population of *melas* falls to a considerably lower level during the period of heavy rains, but rises again as the rains fade away, showing a maintenance of production on a fairly high level into the dry season. During the last 10 weeks of the dry season conditions in the *Avicennia* mangrove zone become progressively worse for breeding, so that production quickly falls away to a very low level. The relationship between tide-levels, rainfall and *melas*-population is graphically illustrated in Appendix V; the circumscribed production-zone, with the adjacent isolated village of Aberdeen, is particularly suitable for this purpose.

In conclusion, it may be said that Muirhead Thomson's researches demonstrated to the malariologist the areas within the tidal zone where dense breeding of *melas* could be expected to occur. It is not suggested that *melas* does not breed elsewhere in saline water, but it is reasonably certain that adequate permanent control of the zones described would cause a great reduction in the numbers of *melas* round the Freetown estuary, with particular reference to that period of the year during which *A. gambiae* type production is minimal, namely, November to May inclusive.

Avicennia Mangrove Areas in Relation to Freetown Estuary. Through the co-operation

of the Royal Air Force, observation from the air enabled 'orchards' of *Avicennia* mangrove to be located in the extensive mangrove forests round the estuary. The distinction was one of colour, a variation in the shade of green of the 'carpet' fringing the water-line; this was most evident between January and March, and air photographs (Appendix VI, C, fig. 20) showed that the areas of *Avicennia* mangrove were not extensive in proportion to the general mangrove area. These areas are indicated on the map of the estuary (Appendix I, map 1). Despite the considerable difficulty of access in some cases, each was inspected at various times by Muirhead Thomson and members of the Army malaria laboratory; larvae were found in all those near to which were African dwellings. The 'orchards' producing a great density of *melas* were at Aberdeen, Wellington, Makanshan and on the northern shore. Those at Aberdeen and Wellington were easy of access from Freetown, and, in consequence, the density of the *melas*-population, the *melas* infectivity-rate and the results of control by the exclusion of tidal waters were studied in relation to these large villages.

THE PROPORTION OF *A. gambiae* VAR. *melas* FOUND INFECTED WITH MALARIA

It was decided towards the end of 1942 to include in the routine work of the Army malaria laboratory during 1943 an investigation into the infection-rate of *A. gambiae* var. *melas*. For this purpose Muirhead Thomson arranged that his African assistants should make regular live random collections of mosquitoes by hand-catching with a glass tube in the houses of Wellington village; this village is a large isolated one adjacent to an *Avicennia* mangrove marsh, approximating 150 acres in extent and producing large numbers of *melas* (Appendix VI, C, fig. 21). A smaller number of 4-banded *melas* were collected in a similar way from various other estuary villages. Such regular weekly collections were continued at Wellington throughout the year, except during April, when very few mosquitoes could be found.

Mosquitoes were conveyed to the laboratory on the day of capture in Barraud's cages, and were kept for 48 hours at room-temperature (75–80° F.) and in a humid atmosphere. Individual identification by wing and palp followed, and, if female *A. gambiae*, the mosquito was classified according to palps, with the following results:

(a) 4-banded <i>melas</i>	Random,	353	Selected, 108
(b) 3-banded, being either 'type' or <i>melas</i>	"	1,009	
(c) 'Unidentifiable,' because of damage to the terminal section of the palps	"	533	
Total			2,003

Egg-Laying and Examination. Groups (b) and (c), namely, the 3-banded and the 'unidentifiable' *A. gambiae*, were then 'set' individually for egg-laying by the simple method of the use of a series of 1½ in. × 1 in. glass tubes, netted at one end and resting on filter-paper moistened with 50 per cent. sea-water in Petri dishes. Room-temperature and high humidity were maintained. By the next or the second morning, eggs had been laid or the mosquito was dead. Eggs were examined microscopically, and so identification of that mosquito-collection was completed. Results were as follows:

	Total	<i>Melas</i>	'Type'	Nil result
3-banded <i>A. gambiae</i>	1,009	486	26	497
'Unidentifiable' <i>A. gambiae</i>	533	133	0	400

*Ratio of 4-Banded to 3-Banded *A. gambiae* var. *melas* Females.* Through the year the collection of mosquitoes was made from the same group of houses in Wellington

village within close range of the *Avicennia* mangrove breeding-zone, and was subjected to approximately constant conditions and treatment in the laboratory, so that it may be assumed that in the egg-laying described in the last paragraph the proportion of 'nil result' 3-banded *A. gambiae*, which were in fact *melas*, was similar to the proportion which laid eggs identified as *melas*.

3-banded *A. gambiae*: total 1,009; proportion *melas*: 'type' = 486:26.

Therefore, total *melas*: $1,009 \times \frac{486}{486 + 26} = 958$.

In random catches from Wellington village the proportion of 4-banded to 3-banded *A. gambiae* var. *melas* was therefore 353:958 = 1:2.7.

This ratio is used in the estimation of numbers of *melas* in the statistical tables relating to proportions of *melas* and 'type' occurring at catching-stations (Appendix III).

Results of Dissection. The numbers of *melas* available for dissection after identification were:

4-banded random catches at Wellington	353
4- " selected as such from estuary villages	108
3- " random catches at Wellington, identified by egg	486
Damaged-palp catches at Wellington, identified by egg	133
Total	1,080

Deaths in the period between identification and dissection further reduced this number, so that, of the original 2,003 *A. gambiae* received in the laboratory during the calendar year, 1,000 *melas* were examined for infection of the salivary glands. The day of dissection after capture varied as follows: 2nd day, 4; 3rd day, 448; 4th day, 381; 5th day, 154; 6th day, 13.

Tables of the sporozoite-rate, the oöcyst-rate and the total infection-rate, showing dissection results month by month, are contained in Appendix II. The summarized results are as follows:

Rate	No. examined	No. positive	Percentage positive
Sporozoite ...	1,000	42	4.2
Oöcyst ...	931	44	4.7
Total infection	885	69	7.8

The summarized results for 4-banded, as compared to 3-banded, *melas* are as follows:

Rate	No. examined	No. positive	Percentage positive
Sporozoite: 4-banded	423	19	4.5
3- "	452	19	4.2
Oöcyst: 4- "	380	21	5.5
3- "	439	18	4.1
Total infection: 4- "	371	33	8.9
3- "	409	30	7.3

It is evident from these results that *A. gambiae* var. *melas* is an effective vector of malaria on the shores of Freetown estuary, comparable in efficiency to *A. gambiae* type and *A. funestus*; details of past results for African vectors are given in Appendix II, Table IV.

The relationship between the monthly sporozoite-rate and the monthly aggregate

of numbers of *melas* caught once per week at one well-placed catching-station at Wellington is shown in graph B of Appendix IV.

Graph A of Appendix IV illustrates the *melas* infective density in the part of Wellington village adjacent to the *melas* production-zone of mangrove; this is an index of the intensity of transmission of malaria, and the graph shows that in June there was present an average of 13 infected *melas* per 10 rooms, in November six per 10 rooms, and in April one per 20 rooms.

THE DENSITY OF *A. gambiae* VAR. *melas* POPULATION

It is necessary to have a clear conception of the density of population of any one vector anopheline in relation to the total vector anopheline population, so that its degree of importance as a transmission-agent may be assessed and measures of control instituted accordingly.

Along the southern shore of Freetown estuary in the wet season *A. gambiae* distribution is immense and universal; it obscures other anopheline production almost completely, and vastly exceeds all culicine production. The other vector anophelines, though in themselves good transmitters of malaria, are localized in their breeding-habits and distribution; *A. funestus* is included in this category.

A. gambiae, *A. funestus*, *A. nili*, *A. hancocki* and *A. hargreavesi* are natural frequenters of human habitations by night, and the dark, dilapidated African dwelling serves also as an excellent day resting-place. A regular census of the mosquito-population in selected resting-places renders an appreciation of the degree of and changes in density of the vector anopheline invaders possible. Mosquito catching-stations were instituted at selected places along the southern shore of the estuary; the statistics from some 50 of these stations are contained in the tables in Appendix III. An individual catching-station is a room or rooms in an African dwelling; each room is 'flitted' with pyrethrum spray by hand at the same time (preferably early morning) on the same day of each week of the year; the mosquitoes fall on to white sheets placed on the floor, and are readily recoverable for counting and removal to the laboratory.

Relevant to the requirements of this investigation, the following information has been tabulated and summarized in groups of catching-stations situated in town, village or district for each month of 1943:

- (A) Total vector anophelines.
- (B) Number of rooms examined.
- (C) Anopheline room-index, i.e., (A) divided by (B).
- (D) *A. gambiae*: identified on general characters.
- (E) Other vector anophelines.
- (F) *A. gambiae*: number with undamaged palps contained in (D).
- (G) Proportion of 4-banded palps in (F).
- (H) Estimated number of *melas* in (F), i.e., (G) multiplied by 3.7; this figure is the

result of the investigation into the ratio of 4-banded to 3-banded *melas* described in the preceding section of this paper.

H

- (K) Numbers of *melas* in (D), i.e., $D \times \frac{H}{F}$

- (L) Numbers of 'type' in (D), i.e., D minus K.

- (M) Vector anopheline percentages.

The deductions from these tables are illustrated graphically in Appendix III by two histograms in relation to each table or locality. One indicates the average vector anopheline room-index for each month; the scale of this histogram is the same for each locality, so that variations in density of mosquito-populations are rendered obvious. The second histogram compares the gross numbers of 'type,' *melas* and 'others' caught each month in the same localities.

Assessment of Statistical Data, 1943

Southern Shore. Reference to the detail given in the tables and the associated histograms in Appendix III elicits the following information:

1. The predominant anopheline is *A. gambiae* type, with greatest density of production in June and July.
2. From May to September the production of *melas* rises in proportion to 'type,' forming between one-quarter and one-third of the total anopheline production; from September to January *melas* maintains its production-level while 'type' falls, so that in November and December the summarized totals of *melas* exceed those of 'type.'
3. The production of 'other' vector anophelines is by comparison insignificant.
4. *Melas* is found in great numbers in the vicinity of circumscribed breeding-grounds; the level of wet-season production is continued into the first half of the dry season; during November, December and January infiltration of villages occurs in the upper reaches of the estuary creeks.
5. *Melas* production is intense where blood-meals are readily available.
6. *Melas* does not normally invade the urban area of Freetown.

Northern Shore. Muirhead Thomson (1944) found that 90 per cent. of anophelines caught in the coastal villages were *melas* and that the density and extent of *melas*-breeding in the circumscribed *Avicennia* mangrove zones was comparable to that at the similar zone at Wellington on the southern shore.

METEOROLOGICAL DATA

Appendix IV, graphs C and D, indicate the monthly averages of temperature, relative humidity and rainfall.

Janscó (1905) quotes 61° F. as the minimum temperature at which the process of sporogony can continue in the mosquito; optimum conditions exist between 77° and 86° F.; above 95° F. conditions become less favourable. The temperature-range through the year on the Freetown estuary shows little variation, and falls within the gambit of optimal conditions for the extracorporeal phase of the life-cycle of *Plasmodium falciparum* in the insect host.

The duration of life of the mosquito is influenced markedly by humidity and temperature, the most favourable conditions being moderate temperature and high humidity. Necheles (1925) considered a relative humidity of 75-80 per cent., with temperature not exceeding 65° F., as optimal, and Gill (1921) quoted a minimum of 63 per cent. (8 a.m. readings) mean monthly relative humidity as necessary for mosquito survival through the sporogonous cycle in India.

During the season of minimal rainfall (January to March) on the Freetown estuary a 'drying' wind (Harmattan) from the north-east causes a comparatively low relative

humidity (65 per cent. at 14.00 hours) coincident with the highest temperatures of the year (mean maximum 87° F.).

The conclusion is that temperature and humidity are unfavourable between January and April for *A. gambiae* var. *melas*; variations in density of production are related to tides, and heavy increase to high spring tides plus moderate rainfall; the peak of production is in June, when the mean monthly relative humidity at 07.00 hours is 92 per cent. and the mean minimum temperature 76° F.

PERMANENT METHODS OF CONTROL OF THE BREEDING OF *ANOPHELES GAMBIAE* VAR. *MELAS*

REMOVAL OF THE SOURCE OF BLOOD-SUPPLY

Under certain favourable geographical conditions (e.g., a village at the end of a peninsula), the removal of the inhabitants of the district within a two-mile radius of a *melas* breeding-zone will cause a large decrease of breeding within that zone, almost to the point of elimination, and will probably render the under-mentioned 'bund'-control unnecessary. This is a useful short-term method of control for military purposes and was used in two instances on the southern shore of the estuary. In 1941 mosquito-catches in the villages concerned showed a room-index of between 20 and 40 anophelines in the wet season; the number of 4-banded *A. gambiae* indicated that 90 per cent. were *melas*; the villages were removed early in 1942. In 1943 very few larvae could be found in the respective *Avicennia* mangrove marshes; adult mosquito-catches in a suitable hut, occupied by Europeans, were small, and, in the absence of Africans, it is probable that few, if any, were infected with malaria.

CONTROL OF *melas*-BREEDING BY EXCLUSION OF TIDAL WATERS FROM BREEDING-ZONES

It has been demonstrated that *A. gambiae* var. *melas* prefers to lay its eggs in saline waters located in the upper parts of the tidal area, and that complete development of the larval and pupal stages occurs in sea-grass and *Avicennia* mangrove zones and not in *Rhizophora* mangrove zones. The principle of control was, therefore, the exclusion of tidal waters from the sea-grass and *Avicennia* marsh zones, in which *melas*-breeding in some considerable density had been found to occur. For experimental purposes the mangrove-lined Aberdeen Creek, situated at the western extremity of the southern shore of Freetown estuary and isolated from other mangrove areas by a distance of eight miles, and the adjacent isolated Aberdeen village were suitable for control and assessment of results.

Aberdeen Creek (Appendix I, map 2)

This creek is two miles in length and one mile wide at the centre, and has a narrow inlet from the estuary. The eastern shore lies at the foot of a spur of the hills which encircle Freetown; the ridge varies between 200 and 500 feet in height and is an effective barrier to the flight of mosquitoes to the east. At the northern half of the creek the western shore flanks the Aberdeen peninsula, which is hilly, the highest point being 250 feet; at the foot of this hill lies Aberdeen village, with about 1,500 inhabitants. The southern half of this shore is a low-lying sand-bar separating the mangrove from a long clear beach swept by Atlantic 'rollers.' The eastern fringe of the creek is rock-bound, with a narrow

belt of *Rhizophora* mangrove, and the western fringe has a narrow belt of sea-grass from near the village to the southern end of the sand-bar and a mangrove zone with a maximum depth of 600 yards. This is *Rhizophora* mangrove, except for an *Avicennia* 'orchard,' the area of which would approximate 100 acres.

Heavy *melas*-breeding occurred in the sea-grass belt from the village end as far as the southern limit of the *Avicennia* mangrove and in a boggy patch at the southern end; larvae were found in the depths of the *Avicennia* 'orchard' but not in *Rhizophora* mangrove. Fresh-water seepages were present in the wet season immediately adjacent to the high-tide line along the peninsula from the village to the commencement of the sand-bar and contained the larvae of *A. gambiae* type; a fresh-water swamp, a prolific breeding-place for 'type,' existed in 1940 close to the village and was permanently drained in that year.

Control by Exclusion of Tidal Water. During the dry season, 1942-43, the northern portion of the sea-grass belt, which was bounded by *Rhizophora* mangrove, was cut off from the tidal waters of the creek by the building of an embankment with manual labour; the height and width in cross-section were dependent on the height of the highest spring tide in relation to the position of the 'bund'; the soil for the construction was obtained from a wide and shallow ditch internal to the embankment and cut on a perimeter line through the fringe of the mangrove adjacent to the sea-grass belt. An intercepting drain was cut along the line of seepages to connect with the perimeter drain. The latter acts as a sump for the cut-off area and is drained at its lowest point through a concrete sluice-gate protected by a tidal flap (Appendix I, diagram 4, and Appendix VI, C, figs. 15-18).

During the dry season, 1943-44, the southern portion of the *melas*-producing sea-grass belt and the adjoining *Avicennia* mangrove were treated in a similar manner, the position of the embankment being along a trace cut to shut off two-thirds of the *Avicennia* 'orchard,' i.e., to include the potential egg-laying zone (Appendix I, diagram 3).

The work on the larger *Avicennia* 'orchard' at Wellington was commenced during the same dry season, a mechanical ditcher being used. The amount of drainage-ditching necessary within the cut-off area is dependent on the rainfall and the amount of fresh water entering the area in the wet season; the basic perimeter and intercepting drains above referred to are essential.

Officials of the Forestry Department expressed the view that, following the exclusion of the sea-water, the *Avicennia* mangrove-trees would die off in a few years and the reclaimed area might be used for agricultural purposes.

Results. In the dry season *melas*-breeding is not possible in the cut-off areas because of absence of water. In the wet season observation was maintained on the first experimental area at Aberdeen, and no *melas*-breeding was found where previously it had been abundant. Pool-formation occurred owing to two factors—heavy rain and the damming back of water resulting from too small a sluice exit. Immediately adjacent to the previous high spring-tide level the fresh-water seepages caused some pool-formation; 'type' bred freely in these pools, but *melas* eggs or larvae were not found. 'Type' showed no tendency to invade the pools in the sea-grass belt, but such pooling must be obviated by adequate drainage and sluice outlets. Breeding in pools in the perimeter drain was not observed and was considered unlikely in the presence of larva-eating fish. *Melas* larvae were observed in man-made pools adjoining and external to the embankment in the

Avicennia mangrove; it is important, therefore, to avoid interference with the swamp surface external to the embankment during construction.

The effect of control-measures on the *A. gambiae* type and var. *melas* population of Aberdeen village may be studied in the mosquito-catching-station records for the years 1941, 1942 and 1943, summarized in the tables and histograms in Appendix III, E. During the three years adult-mosquito destruction was practised in the village; the breeding-places of *A. gambiae* type were unusually clearly defined in the fresh-water swamp and seepage belt above referred to; 'permanent' control of the swamp had been effected in 1941; complete control of swamp and seepages was maintained in 1942 by drainage and larvicides; in 1943 larvicides were not used. *Melas*-breeding was uncontrolled until the dry season of 1942-43, when the northern section of the sea-grass belt near the village was controlled by the exclusion of tidal water; the southern section with the adjoining *Avicennia* mangrove remained uncontrolled during 1943. The following extract from the tables of mosquito-catches in Aberdeen village (Appendix III, E) indicates the very considerable decrease in the *melas*-population resulting from the control of that section of the breeding-zone nearest to the village.

Year	<i>Anopheles gambiae</i>	Anopheline room-index*				
		June	July	Aug.	Sept.	Oct.
1941	'Type' plus <i>melas</i>	25.3	19.2	14.2	9.6	1.3
1942	<i>Melas</i>	5.9	14.9	10.1	10.1	6.7
	'Type'	Nil	Nil	Nil	Nil	Nil
1943	<i>Melas</i>	3.0	3.0	1.5	2.2	1.1
	'Type'	2.8	2.5	2.0	0.7	0.3

* Anopheline room-index = Average number of mosquitoes per room per day.

Conclusion. The results of the experiment at Aberdeen justified the application of this method of *melas*-control to other similar breeding-zones on the shores of Freetown estuary; the malariologist investigates and decides which zones require control and the approximate position of the embankments; the malaria engineer constructs the embankment and the requisite drainage and sluice-gates; later, the agriculturalist, in collaboration with the malariologist, develops the reclaimed area on lines which will prevent its conversion into a prolific *A. gambiae* type breeding-ground.

SUMMARY

1. A brief appraisal of the malaria situation in the vicinity of the shores of Freetown estuary is given in relation to the period during which the strategic importance of Freetown in the conduct of the war was at its height.
2. That *A. gambiae* var. *melas* is an efficient vector of malaria is proved.
3. The principal breeding-places of *A. gambiae* var. *melas* are indicated.
4. Statistical data provide evidence of the comparative densities of *A. gambiae* type and *A. gambiae* var. *melas* which invade human dwellings on the southern shore of Freetown estuary.
5. A permanent method of control is described.

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Mr. H. Macluskie, of the Sierra Leone Government Agricultural Department, demonstrated the results of a sea-grass-*Avicennia* marsh reclamation scheme for rice cultivation, which had been in operation for three years. Major P. X. Bermingham, of an Army Field Hygiene Section, supervised the construction of the embankment at Aberdeen; the plans for the sluice-gate were drawn by the Civil Irrigation Engineer, Mr. F. Rae.

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APPENDICES

APPENDIX I

Map 1. General map of Freetown estuary.

Map 2. Aberdeen Creek.

Diagram 1. (a) The palp of the female *A. gambiae* type; (b) the palp of *A. gambiae* var. *melas* female.

Diagram 2. The egg of *A. gambiae* type and of *A. gambiae* var. *melas*.

Diagram 3. The position of the embankment excluding the tide from *melas* egg-laying zones.

Diagram 4. Diagrammatic cross-section of the perimeter drain and the embankment.

APPENDIX II

Table I. Sporozoite-rate.

Table II. Oöcyst-rate.

Table III. Total infection-rate.

Table IV. Records of previous dissections of African vector anophelines.

APPENDIX III

A. Statistical tables.

B. Commentary.

C. Histograms of anopheline room-indices.

D. Histograms of monthly aggregates of routine catches of female vector anophelines.

E. Aberdeen village. Information covers a period of three years and illustrates the effect of control-measures on routine anopheline catches.

APPENDIX IV

Graph A. Infective density of *A. gambiae* var. *melas* at Wellington, 1943.

Graph B. Sporozoite-rate in relation to *melas* density at Wellington, 1943.

Graph C. Temperatures and relative humidity at Freetown, 1943.

Graph D. Rainfall at Freetown, 1943.

APPENDIX V

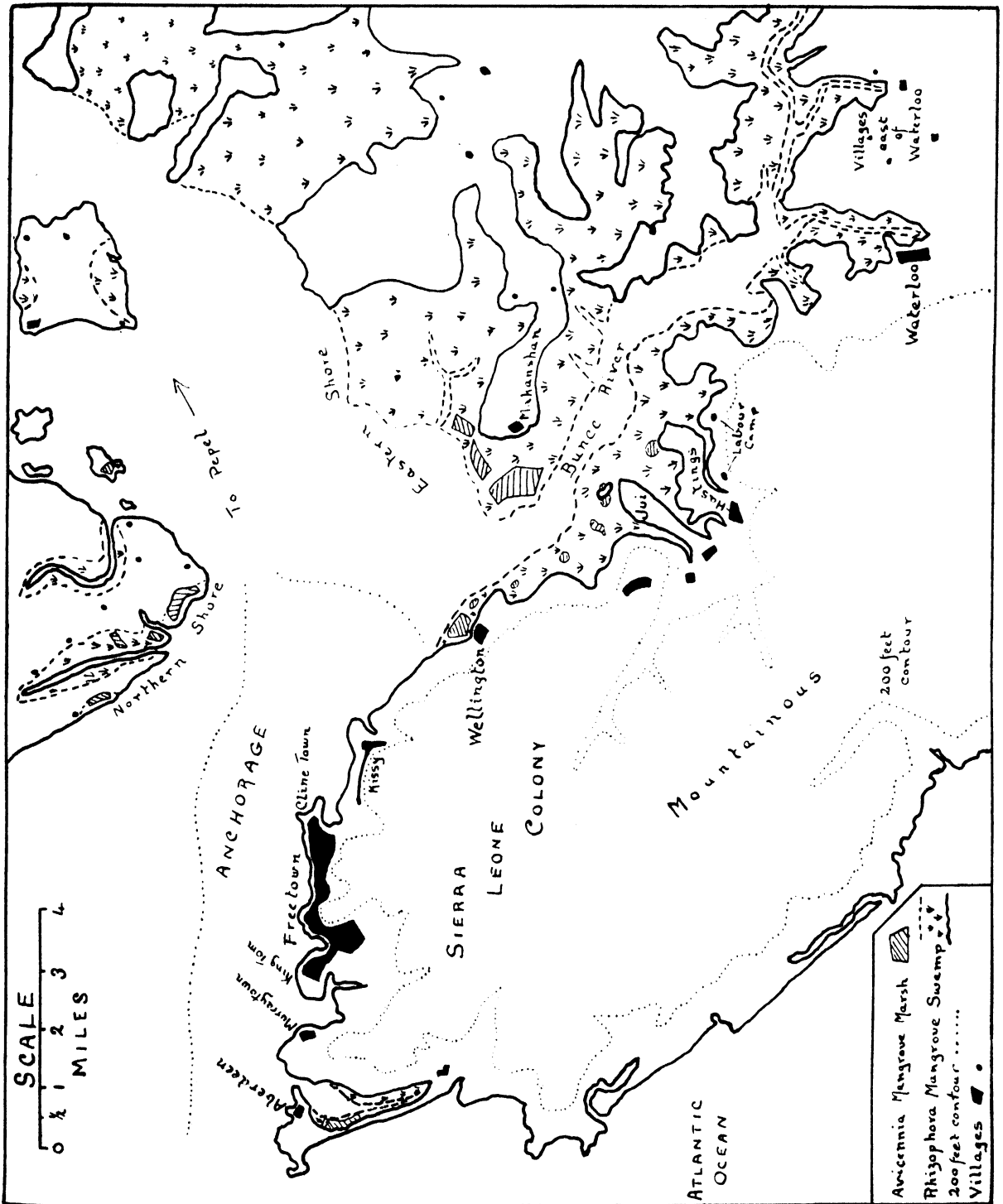
Histogram illustrating relationship at Aberdeen between (a) tides which flood the egg-laying zones; (b) *A. gambiae* var. *melas* room-indices in the adjacent village; (c) daily rainfall; (d) tide exclusion by embankment construction.

APPENDIX VI

A. Diagrammatic cross-sections of *A. gambiae* var. *melas* breeding-zones.

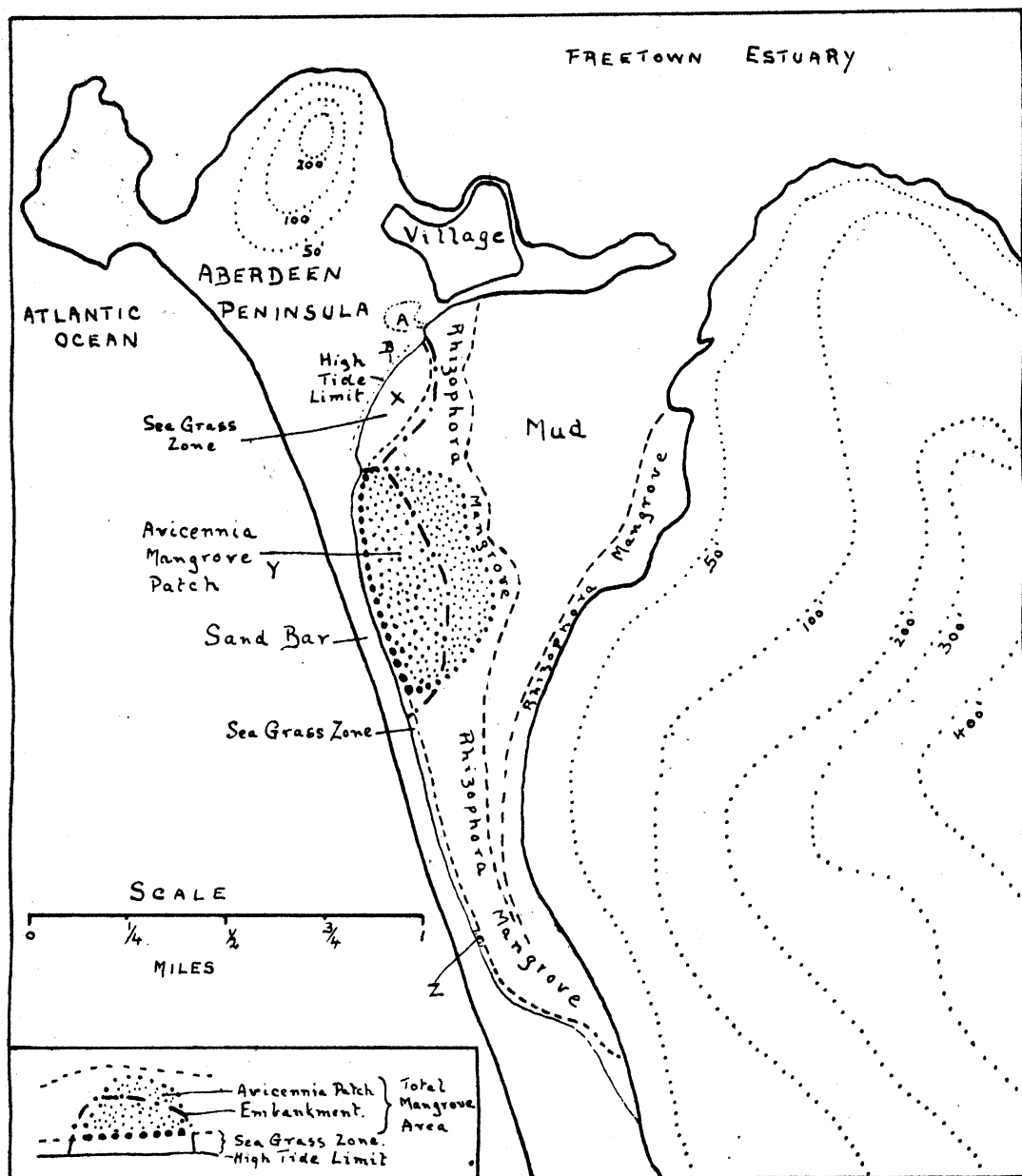
B. Description of *Rhizophora* and *Avicennia* mangrove and the sea-grass belt.

C. Annotated photographs, figs. 1-21.



MAP 1. General map of Freetown estuary.

APPENDIX I (CON.)

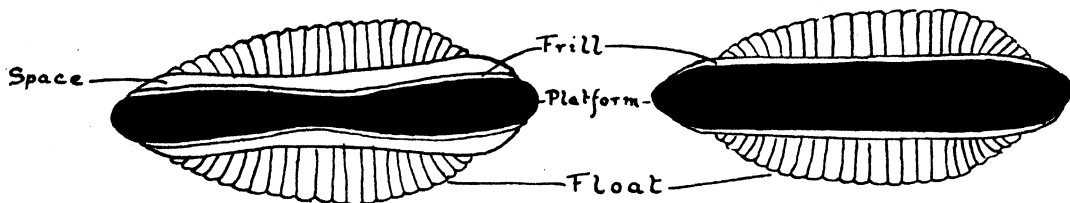


MAP 2. Aberdeen Creek.

DIAGRAM 1 (a). The palp of the female *A. gambiae* type : three pale bands.



DIAGRAM 1 (b). The palp of *A. gambiae* var. *melas* female ; showing the variation from a completely pale apical band (3-banded *melas*) to the division into two pale bands (4-banded *melas*).



A. gambiae type : space present.

A. gambiae var. *melas* : space absent.

DIAGRAM 2. The egg.

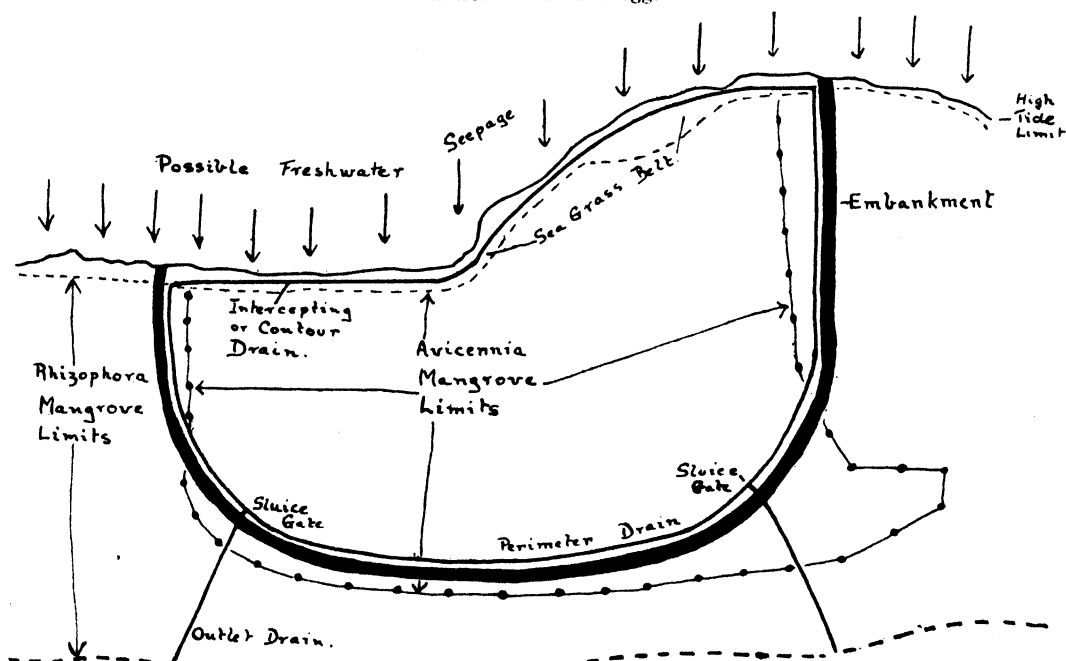


DIAGRAM 3. The position of the embankment excluding the tide from *melas* egg-laying zones.

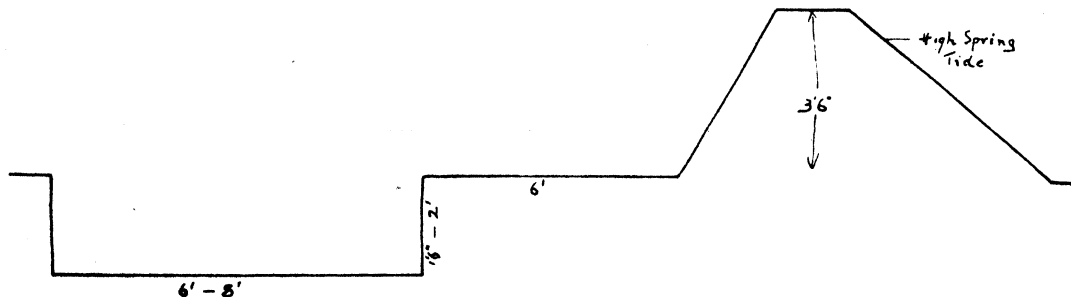


DIAGRAM 4. Diagrammatic cross-section of the perimeter drain and the embankment. The measurements shown are average ; the height of the embankment at any one point is nine inches to one foot above the level of the highest spring tide.

APPENDIX II

TABLE I

Sporozoite-rate

A. gambiae var. *melas* : identified 4-banded forms, 3-banded forms identified on egg character, and females with destroyed or damaged palps identified on egg character

Month	No. of glands examined	No. of glands positive	Percentage positive
January ...	98	0	0
February ...	71	1	1.4
March ...	52	7	13.5
April ...	Nil	Nil	Nil
May ...	64	3	4.7
June ...	113	4	3.5
July ...	130	4	3.1
August ...	124	5	4.0
September ...	79	3	4.0
October ...	112	6	5.4
November ...	75	4	5.3
December ...	82	5	6.1
Total ...	1,000	42	4.2

TABLE II

Oöcyst-rate

A. gambiae var. *melas* : identified 4-banded forms, 3-banded forms identified on egg character, and females with destroyed or damaged palps identified on egg character

Month	No. of stomachs examined	No. of stomachs positive	Percentage positive
January ...	96	0	0
February ...	58	1	1.7
March ...	53	6	11.3
April ...	Nil	Nil	Nil
May ...	58	5	8.6
June ...	106	2	1.9
July ...	126	4	3.2
August ...	100	7	7.0
September ...	75	3	4.0
October ...	109	8	7.3
November ...	72	3	4.0
December ...	78	5	6.4
Total ...	931	44	4.7

APPENDIX II (CON.)

TABLE III

Total infection-rate

A. gambiae var. *melas*: identified 4-banded forms, 3-banded forms identified on egg character, and females with destroyed or damaged palps identified on egg character, examined for both sporozoites in the salivary glands and oöcysts in the stomach

Month	No. of mosquitoes examined	No. positive (sporozoites and/or oöcysts)	Percentage positive
January ...	83	0	0
February ...	49	2	4.1
March ...	50	9	18.0
April ...	Nil	Nil	Nil
May ...	57	6	10.5
June ...	102	6	5.9
July ...	119	6	5.0
August ...	97	11	11.3
September ...	73	5	6.8
October ...	105	11	10.5
November ...	72	5	6.9
December ...	78	8	10.3
Total ...	885	69	7.8

TABLE IV

Records of previous dissections of African vector anophelines

Species	Investigator	Place	Date	No. dissected	Oöcyst-rate	Sporozoite-rate
<i>A. gambiae</i>	Ross, Annett and Austen	Freetown, Sierra Leone	1900	109	19.3	5.5
	Wood	Koinadugu, Sierra Leone	1915	91	7.2	8.7
	Gordon <i>et al.</i>	Kissy, Sierra Leone	1932	1,157	5.2	11.2
	Gordon <i>et al.</i>	Freetown, Sierra Leone	1932	1,156	3.2	8.0
	Davis	Brazil	1930	172	59.3	30.2
	Causey	Brazil	1940	1,891	4.7	1.6
	Mackelvie	Marampa, Sierra Leone	1933-34 1934-35	1,006 4,454	11.5 7.7	13.0 7.9
<i>A. funestus</i>	Gordon <i>et al.</i>	Kissy, Sierra Leone	1932	908	2.9	4.1
	Mackelvie	Marampa, Sierra Leone	1935	158	5.1	5.1
<i>A. nili</i>	Gordon <i>et al.</i>	Kissy, Sierra Leone	1932	22	18.2	9.1
<i>A. hancocki</i>	Gibbins	Uganda	1932	1,014	?	2.7
<i>A. hargreavsi</i>	Barber and Olinger	Lagos, Nigeria	1931	92	2.2	5.4

Summary of catches of female vector anophelines at weekly intervals at catching-stations

FREETOWN URBAN AREA

Month, 1943	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
A. Total vector anophelines	71	72	218	84	80	110	96	62	82	65	17	10	987
B. Rooms examined	292	296	379	297	305	344	294	350	368	348	416	369	4,058
C. Anopheline room-index — B	0.24	0.24	0.58	0.28	0.26	0.32	0.33	0.18	0.22	0.19	0.04	0.03	0.24
D. <i>A. gambiae</i>	71	72	217	83	80	109	96	62	79	56	17	10	952
E. Other vector anophelines	—	—	1	1	—	1	—	—	3	9	—	—	15
F. <i>A. gambiae</i> —undamaged palps	70	67	188	83	80	109	96	62	79	56	17	10	917
G. Proportion 4-banded in F.	1	—	—	—	—	2	—	2	—	—	—	—	5
H. Estimate <i>melas</i> in F. $G \times 3.7$	4	—	—	—	—	7	—	7	—	—	—	—	18
K. <i>A. gambiae</i> { $\frac{H}{D \times \frac{H}{F}}$ <i>melas</i>	4	—	—	—	—	7	—	7	—	—	—	—	18
L. { type D—K	67	72	217	83	80	102	96	55	79	56	17	10	934
Vector anopheline percentages { $\frac{L}{A} \times 100$ <i>A. gambiae</i> type	94	100	100	99	100	93	100	89	96	86	100	100	97
{ $\frac{K}{A} \times 100$ <i>melas</i>	6	—	—	—	—	6	—	11	—	—	—	—	2
{ $\frac{E}{A} \times 100$ other	—	—	—	1	—	1	—	—	4	14	—	—	1

STATISTICAL TABLES

Summary of catches of female vector anophelines at weekly intervals at catching-stations

KISSY VILLAGE AREA

	Month, 1943	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
A.	Total vector anophelines	12	8	20	18	73	832	625	211	274	125	44	21	2,263
B.	Rooms examined	62	62	89	102	85	94	80	92	92	112	95	72	1,037
C.	Anopheline room-index	0.19	0.13	0.23	0.18	0.86	8.86	7.81	2.29	2.97	1.12	0.46	0.29	2.18
D.	<i>A. gambiae</i>	12	8	9	12	60	815	611	190	245	111	42	19	2,134
E.	Other vector anophelines	—	—	11	6	13	17	14	21	29	14	2	2	129
F.	<i>A. gambiae</i> —undamaged palps	12	8	9	12	60	773	584	190	245	111	42	19	2,065
G.	Proportion 4-banded in F.	—	—	—	—	1	7	3	7	3	—	2	—	23
H.	Estimate <i>melas</i> in F. $G \times 3.7$	—	—	—	—	4	26	11	26	11	—	7	—	85
K.	<i>A. gambiae</i> { <i>melas</i> $D \times \frac{H}{F}$	—	—	—	—	4	27	12	26	11	—	7	—	87
L.	type $D - K$	12	8	9	12	56	738	599	164	234	111	35	19	2,047
	<i>A. gambiae</i> type $L - \frac{L}{A} \times 100$	100	100	45	67	77	95	96	78	85	89	80	90	90
	Vector anopheline <i>melas</i> { $K - \frac{K}{A} \times 100$	—	—	—	—	5	3	2	12	4	—	16	—	4
	percentages { $E - \frac{E}{A} \times 100$	—	—	55	33	18	2	2	10	11	11	4	10	6

Summary of catches of female vector anophelines at weekly intervals at catching-stations
WELLINGTON VILLAGE

Month, 1943-1944	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	Total
A. Total vector anophelines	43	188	44	36	21	21	53	15	34	57	17	4	533
B. Rooms examined	3	5	4	4	4	4	5	4	4	4	5	2	48
C. Anopheline room-index — A B	14.3	37.6	11.0	9.0	5.2	5.2	10.6	3.7	8.5	14.2	3.4	2.0	11.1
D. <i>A. gambiae</i>	43	184	43	35	19	18	53	15	33	57	17	4	521
E. Other vector anophelines	—	4	1	1	2	3	—	—	1	—	—	—	12
F. <i>A. gambiae</i> —undamaged palps	43	184	43	35	19	18	53	15	33	57	17	4	521
G. Proportion 4-banded in F. ...	11	49	14	9	5	5	16	6	11	20	4	2	152
H. Estimate <i>melas</i> in F. $G \times 3.7$	41	181	43	33	19	18	53	15	33	57	15	4	512
K. <i>A. gambiae</i> { <i>melas</i> type D—K	41	181	43	33	19	18	53	15	33	57	15	4	512
L.	2	3	—	2	—	—	—	—	—	—	2	—	9
Vector anopheline percentages { <i>A. gambiae</i> type $\frac{L}{A} \times 100$ <i>melas</i> $\frac{K}{A} \times 100$ other $\frac{E}{A} \times 100$	5	2	—	6	—	—	—	—	—	—	12	—	2
	95	96	98	92	90	86	100	100	97	100	88	100	96
	—	2	2	2	10	14	—	—	3	—	—	—	2

APPENDIX III, A (CON.)

STATISTICAL TABLES

Summary of catches of female vector anophelines at weekly intervals at catching-stations

HASTINGS VILLAGE AREA

Month, 1943		Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
A. Total vector anophelines ...		13	25	39	22	56	155	68	24	7	10	50	36	505
B. Rooms examined ...		38	37	45	31	40	32	36	45	25	32	37	35	433
C. Anopheline room-index		0.34	0.67	0.87	0.71	1.40	4.85	1.89	0.53	0.28	0.31	0.81	1.03	1.16
D. <i>A. gambiae</i> ...		13	25	39	19	54	154	68	23	7	10	49	35	496
E. Other vector anophelines ...		—	—	—	3	2	1	—	1	—	—	1	1	9
F. <i>A. gambiae</i> —undamaged palps		12	24	39	18	51	95	64	23	5	10	49	35	425
G. Proportion 4-banded in F. ...		—	—	—	—	2	5	1	2	1	1	5	5	22
H. Estimate <i>melas</i> in F. $G \times 3.7$		—	—	—	—	7	19	4	7	4	4	19	19	83
K. <i>A. gambiae</i> { $\begin{matrix} \text{type} & D \times \\ & H \end{matrix}$		—	—	—	—	7	31	4	7	6	4	19	19	97
L. { $\begin{matrix} & D - K \\ & F \end{matrix}$		13	25	39	19	47	123	64	16	1	6	30	16	399
Vector anopheline percentages		100	100	100	86	84	79	94	67	14	60	60	44	79
A. <i>gambiae</i> type		—	—	—	—	12	20	6	29	86	40	38	53	19
other		—	—	—	14	4	1	—	4	—	—	2	3	2

APPENDIX III, A (CON.)

STATISTICAL TABLES

Summary of catches of female vector anophelines at weekly intervals at catching-stations
VILLAGES EAST OF WATERLOO TOWN

Month, 1943	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
A. Total vector anophelines	52	45	41	12	44	181	280	24	45	40	58	47	869
B. Rooms examined	44	46	66	37	47	46	68	55	48	70	54	56	637
C. Anopheline room-index — A	1.18	0.98	0.68	0.33	0.94	3.94	4.11	0.44	0.94	0.57	1.07	0.84	1.36
— B													
D. <i>A. gambiae</i>	52	42	39	12	43	181	279	24	38	37	58	47	852
E. Other vector anophelines	—	3	2	—	1	—	1	—	7	3	—	—	17
F. <i>A. gambiae</i> —undamaged palps	40	35	37	12	43	170	278	24	38	37	58	35	807
G. Proportion 4-banded in F. ...	4	—	1	—	3	9	5	—	1	—	6	1	30
H. Estimate <i>melas</i> in F. $G \times 3.7$	15	—	4	—	11	33	19	—	4	—	22	4	112
K. <i>A. gambiae</i> $\left\{ \begin{array}{l} \text{melas} \\ \text{type} \end{array} \right. \left\{ \begin{array}{l} H \\ D \times F \\ D - K \end{array} \right.$	20	—	4	—	11	35	19	—	4	—	22	5	120
L. $\left\{ \begin{array}{l} A. gambiae \text{ type} \\ \text{Vector anopheline percentages} \end{array} \right. \left\{ \begin{array}{l} L \\ K \\ E \end{array} \right. \left\{ \begin{array}{l} \frac{L}{A} \times 100 \\ \frac{K}{A} \times 100 \\ \frac{E}{A} \times 100 \end{array} \right.$	62	93	85	100	73	81	93	100	76	94	62	89	84
— <i>melas</i>	38	—	10	—	25	19	7	—	9	—	38	11	14
— other	—	7	5	—	2	—	—	—	15	6	—	—	2

APPENDIX III, B

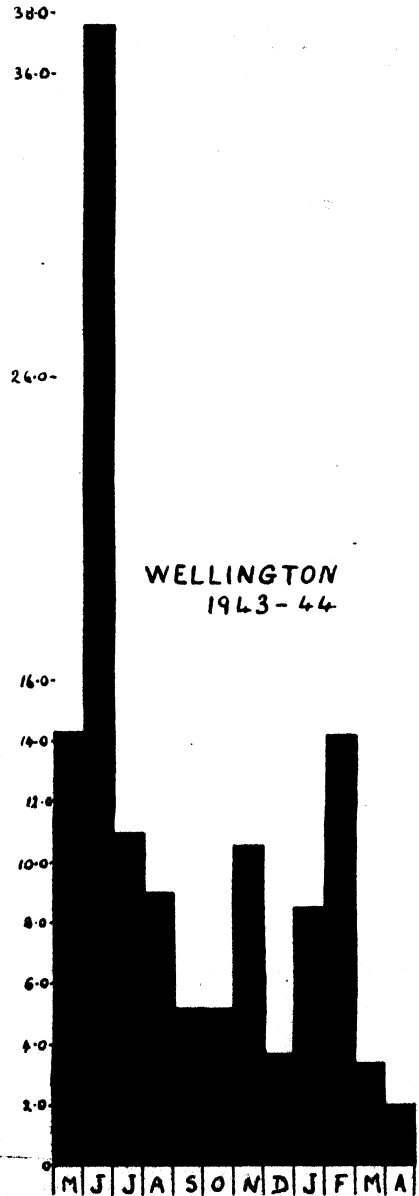
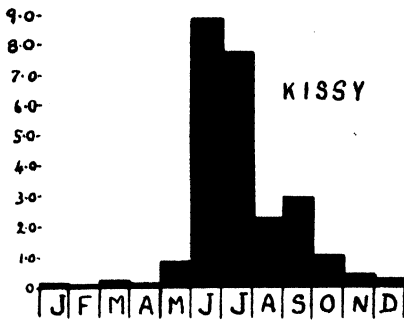
COMMENTARY

District	African population	Catching-stations	Breeding-areas	Control-measures	Services quarters	Comments
Freetown urban area	Approx. 60,000	23	Rocky-bedded streams and seepages.	Adult destruction ; permanent drainage ; larvicides.	Unscreened.	Efficient results dependent on vigilance of African inspectors. March figure indicates 'missed' breeding. Generally few anophelines in central Freetown.
Kissy village area	Approx. 4,000	9	In wet season extensive sunlit pools and seepages.	Adult destruction; drainage ; larvicides.	Metal-gauze screening.	<i>Melas</i> infiltration from east slight. Control ineffective ; road and building construction an important factor.
Wellington village	Approx. 3,000	1	<i>Avicennia</i> mangrove marsh. Fresh-water coastal marsh with seepages.	Drainage of fresh-water marsh, with seepage-control and 'oiling' when necessary.	Metal-gauze screening.	Very heavy <i>melas</i> production. The single catching-station was situated at the centre of the fringe of the village adjoining the salt-water marsh, and is an indication of the density of invasion by <i>melas</i> .
Hastings village area	Approx. 3,000	5	Streams, seepages, and, in the wet season, sunlit pools over a wide area. <i>Avicennia</i> mangrove swamp two miles distant.	Canalization of streams, drainage-control of seepages, and larvicides. <i>Avicennia</i> salt-marsh uncontrolled, but adjacent villagers removed to Hastings town. Adult-mosquito destruction.	Metal-gauze screening.	Some <i>melas</i> infiltration at early rain and post-rain periods.
Hastings labour-camps	Approx. 500	2	Fresh-water seepages and streams on hillside and near shore-line. <i>Avicennia</i> mangrove swamp two miles distant across <i>Rhizophora</i> mangrove creek.	Adult destruction.	Metal-gauze screening.	Situation 100 feet above sea-level, overlooking the upper reaches of the Bunce River. Consistent occurrence of <i>melas</i> through the year, particularly at the early rain and post-rain periods.
Villages east of Waterloo town	Approx. 2,000	7	Swamp (fresh-water), seepages, sunlit pools. <i>Melas</i> - breeding not found.	Adult destruction; swamp drainage ; larvicides in wet season.	Metal-gauze screening.	<i>Melas</i> adults in small numbers near tidal creeks at early rain and post-rain periods.

APPENDIX III, C

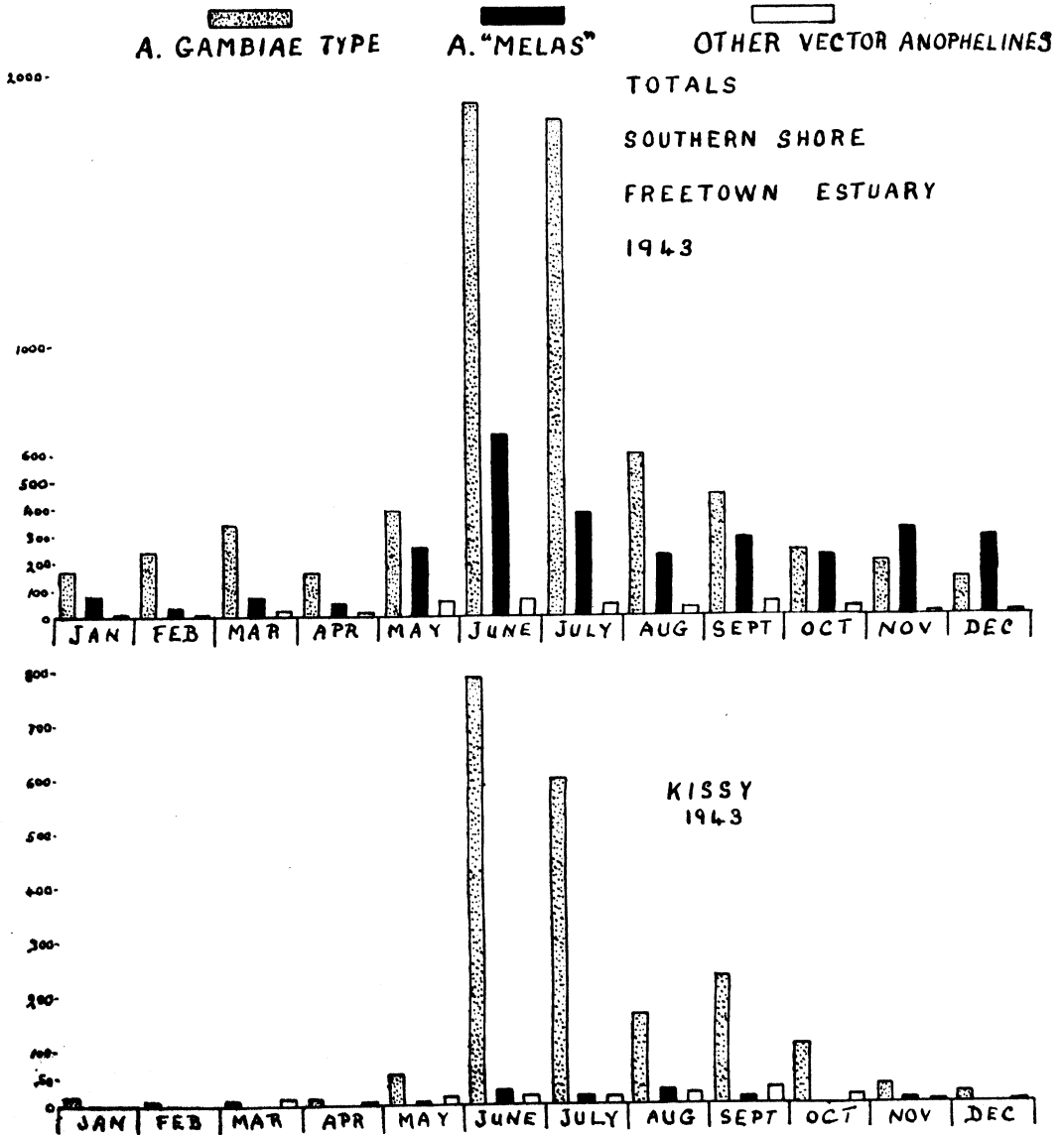
ANOPHELINE ROOM INDICES

1943

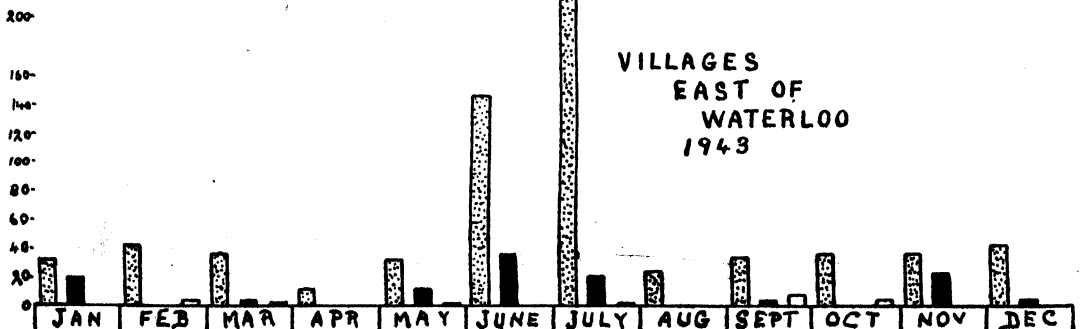
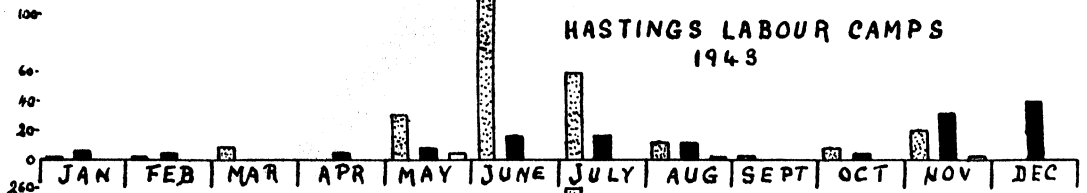
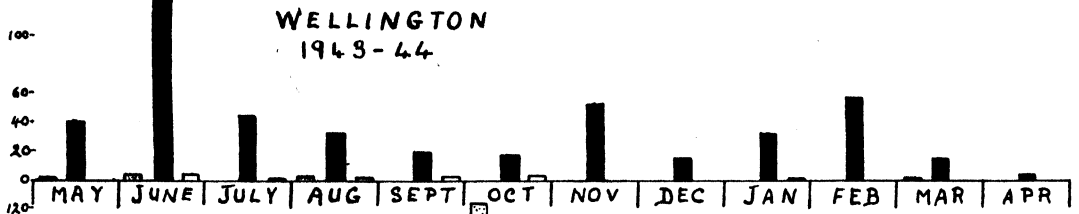
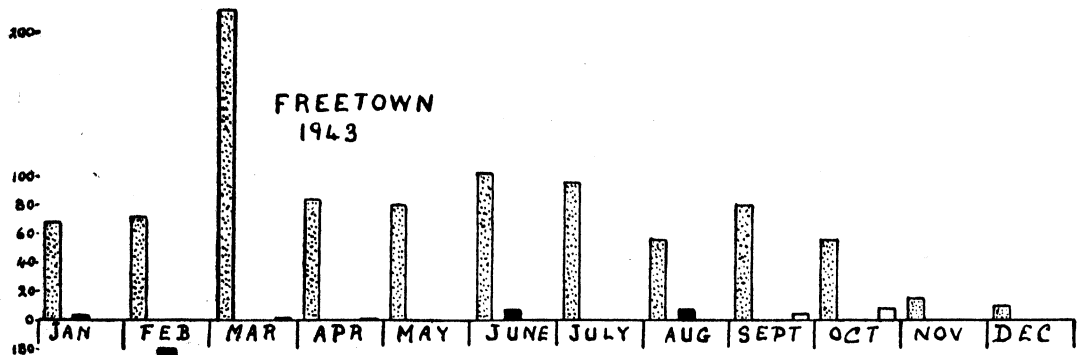


APPENDIX III, D

MONTHLY AGGREGATES OF ROUTINE CATCHES OF FEMALE VECTOR ANOPHELINES



APPENDIX III, D (CON.)



APPENDIX III, E

Summary of catches of female anophelines at weekly intervals at catching-stations

ABERDEEN VILLAGE

Month, 1941		Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
A. Total anophelines	...	429	242	114	36	131	2,102	1,405	1,241	1,036	80	5	22	6,843
B. Rooms examined	...	100	92	84	85	99	83	73	87	108	60	15	50	936
C. Anopheline room-index	A B	4.3	2.63	1.36	0.42	1.32	25.3	19.2	14.2	9.6	1.3	0.33	0.44	7.31
Month, 1942		Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
A. Total vector anophelines	...	99	42	17	38	20	356	1,030	495	383	304	29	40	2,853
B. Rooms examined	...	115	94	104	138	76	60	69	49	38	45	31	30	849
C. Anopheline room-index	A B	0.86	0.45	0.16	0.26	0.26	5.93	14.9	10.1	10.1	6.7	0.94	1.33	3.36
D. <i>A. gambiae</i>	...	99	42	17	38	20	356	1,030	495	383	304	29	40	2,853
E. Other vector anophelines	...	—	—	—	—	—	—	—	—	—	—	—	—	—
F. <i>A. gambiae</i> —undamaged palps	...	79	41	17	33	20	201	886	470	186	276	28	38	2,275
G. Proportion 4-banded in F.	...	12	11	6	15	7	89	354	131	73	83	11	10	802
H. Estimate <i>melas</i> in F.	$G \times 3.7$	44	41	17	33	20	201	886	470	186	276	28	37	2,239
K. <i>A. gambiae</i> { <i>melas</i> type	$\begin{matrix} H \\ D \times \frac{H}{F} \\ D - K \end{matrix}$	55 44	42 —	17 —	38 —	20 —	356 —	1,030 —	495 —	383 —	304 —	29 —	39 1	2,808 45
L. Vector anopheline percentages	$\begin{matrix} A. gambiae \text{ type } \frac{L}{A} \times 100 \\ K \\ \text{other } \frac{E}{A} \times 100 \end{matrix}$	44 56 —	— 100 —	— 100 —	— 100 —	— 100 —	— 100 —	— 100 —	— 100 —	— 100 —	— 100 —	— 100 —	— 97 —	— 98 —

APPENDIX III, E (CON.)

Summary of catches of female anophelines at weekly intervals at catching stations
ABERDEEN VILLAGE (CON.)

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
Month, 1943													
A. Total vector anophelines	10	23	28	19	96	361	353	191	192	76	56	49	1,454
B. Rooms examined	42	43	50	64	62	62	64	54	66	56	46	47	656
C. Anopheline room-index	0.24	0.54	0.56	0.30	1.54	5.82	5.51	3.54	2.91	1.36	1.22	1.04	2.22
D. <i>A. gambiae</i>	10	23	28	19	96	361	353	191	189	74	56	49	1,449
E. Other vector anophelines	—	—	—	—	—	—	—	—	3	2	—	—	5
F. <i>A. gambiae</i> —undamaged palps	10	17	23	12	93	361	353	190	188	74	56	49	1,426
G. Proportion 4-banded in F.	3	6	11	4	32	51	52	21	40	16	16	9	260
H. Estimate <i>melas</i> in F. $G \times 3.7$	10	17	23	12	93	189	192	78	148	59	56	33	910
K. <i>A. gambiae</i> { <i>melas</i> $D \times \frac{H}{F}$	10	23	28	19	96	189	192	78	148	59	56	33	931
L. { type $D - K$	—	—	—	—	—	172	161	113	41	15	—	16	518
Vector anopheline percentages													
<i>A. gambiae</i> type $\frac{L}{A} \times 100$	—	—	—	—	—	48	46	59	21	20	—	33	36
<i>melas</i> $\frac{K}{A} \times 100$	100	100	100	100	100	52	54	41	77	78	100	67	64
other $\frac{E}{A} \times 100$	—	—	—	—	—	—	—	—	2	2	—	—	—

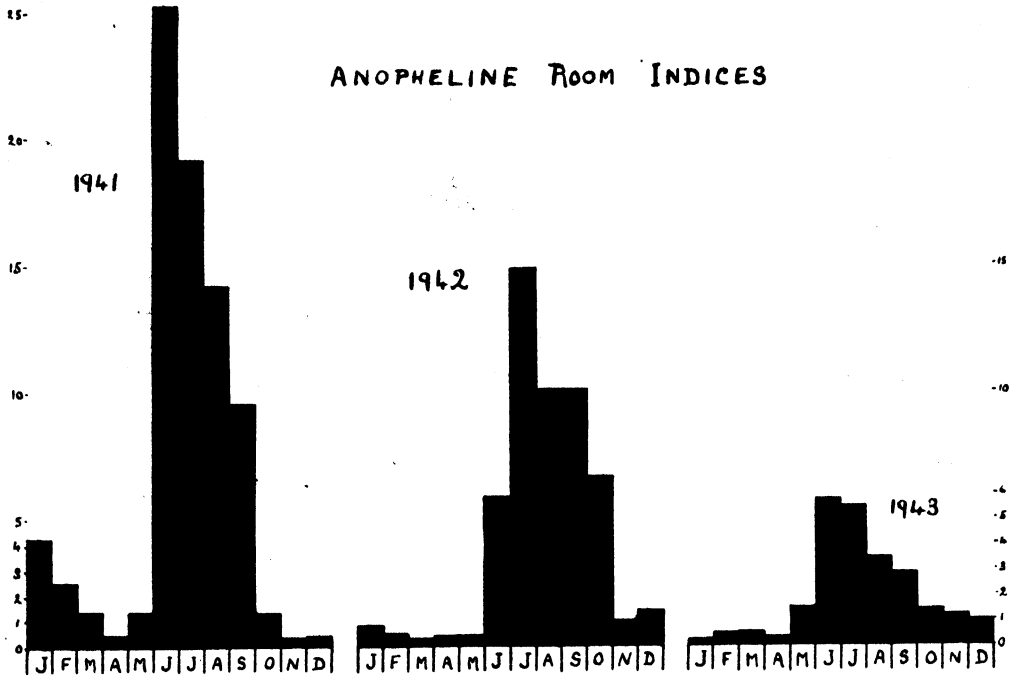
APPENDIX III, E (CON.)

ABERDEEN VILLAGE
Commentary

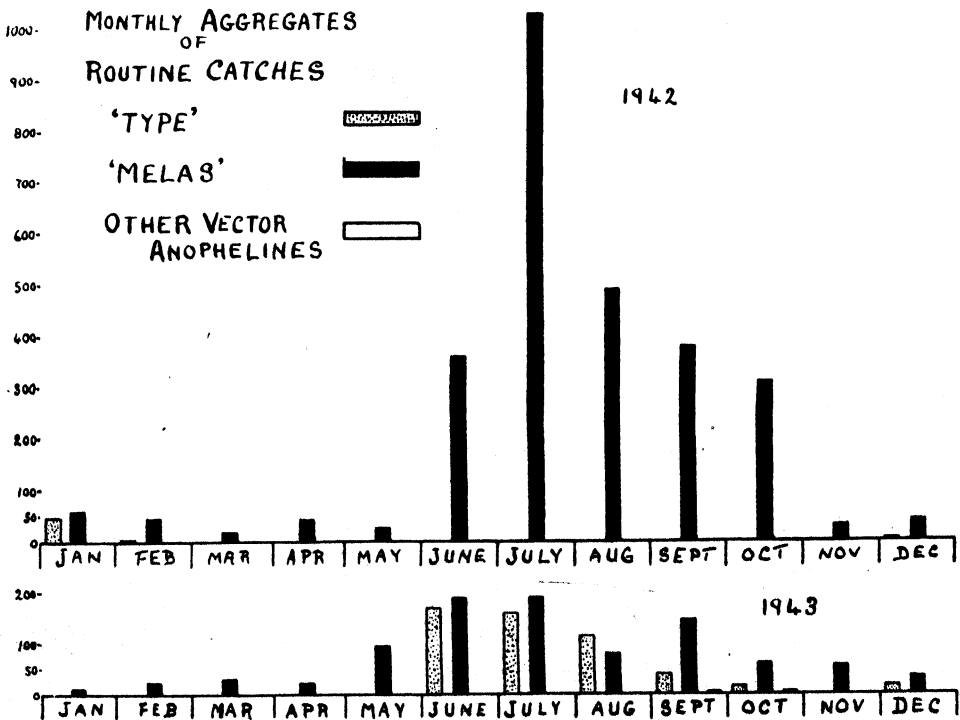
African population	Catching-stations	Breeding-areas	Control-measures	Service quarters	Comments
Approx. 1,500	4-6	<i>A. gambiae</i> type clearly defined as one fresh-water swamp (map 2, A) and a line of seepages (map 2, B) at the foot of a low hill ridge; the remainder of the peninsula is hilly, undulating and self-draining, with little opportunity for sunlit pool-formation in the wet season. <i>Melas</i> area was confined to the sea-grass zone (map 2, X), the <i>Avicennia</i> mangrove zone with adjoining sea-grass belt (map 2, Y), and the distant circumscribed sea-grass 'patch' (map 2, Z).	1941: adult destruction and larvicides for fresh-water breeding. 1942: adult destruction, permanent swamp-drainage, intercepting drain for seepages, and accessory larvicides. 1943: adult destruction, drainage only for fresh-water breeding; exclusion of tidal water from the sea-grass zone (map 2, X); remaining <i>melas</i> zones uncontrolled.	R. A. F. : metal-gauze screening; remainder unscreened.	Originally a heavily infested village by both 'type' and <i>melas</i> ; 'type' readily controllable; <i>melas</i> controllable by the exclusion of tidal water, the partial control effected in the dry season 1942-43 producing the result shown in the 'Anopheline Room - Index' histogram for 1943, the proportion of <i>melas</i> being below the 2.5 line.

APPENDIX III, E (CON.)

ABERDEEN



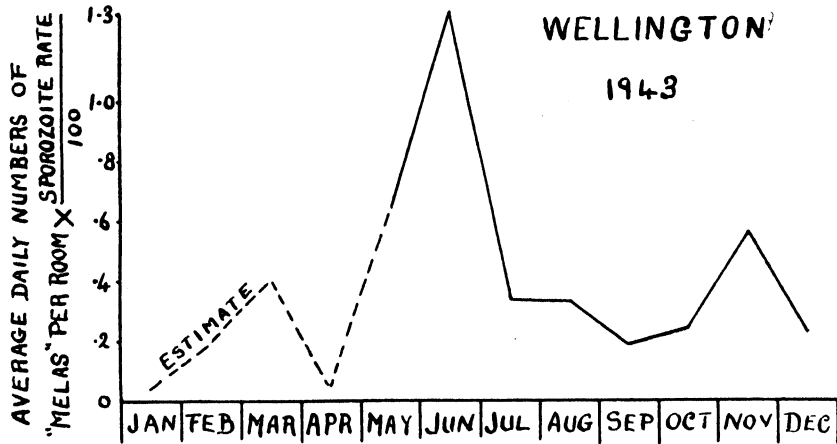
ABERDEEN



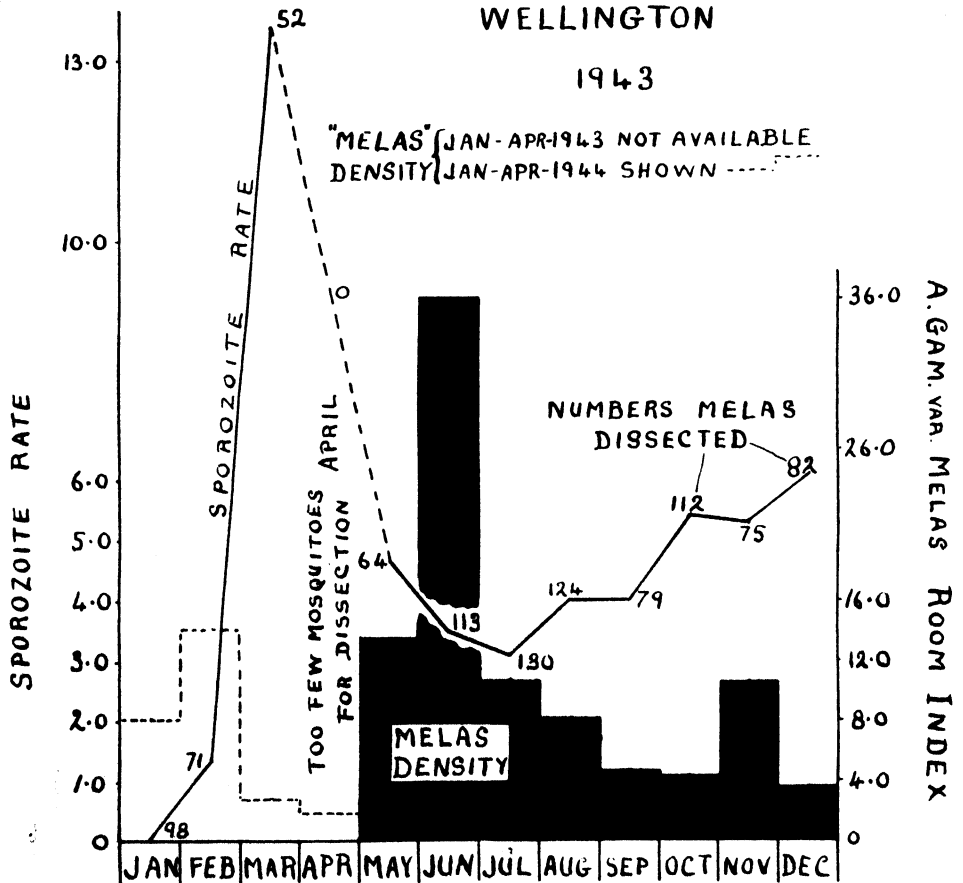
APPENDIX IV

INFECTIVE DENSITY OF A. GAMBIAE VAR MELAS

GRAPH

A

GRAPH

BSPOROZOITE RATE IN RELATION TO "MELAS" DENSITY

APPENDIX IV (CON.)

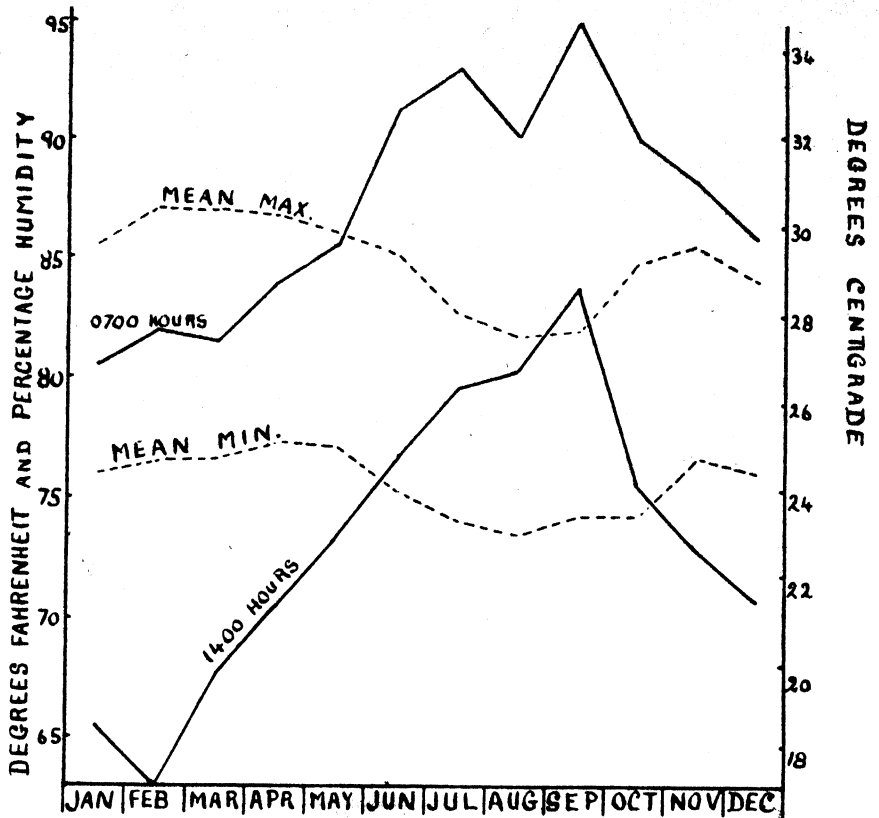
GRAPH C

TEMPERATURES

RELATIVE
HUMIDITY

———

1943

GRAPH D

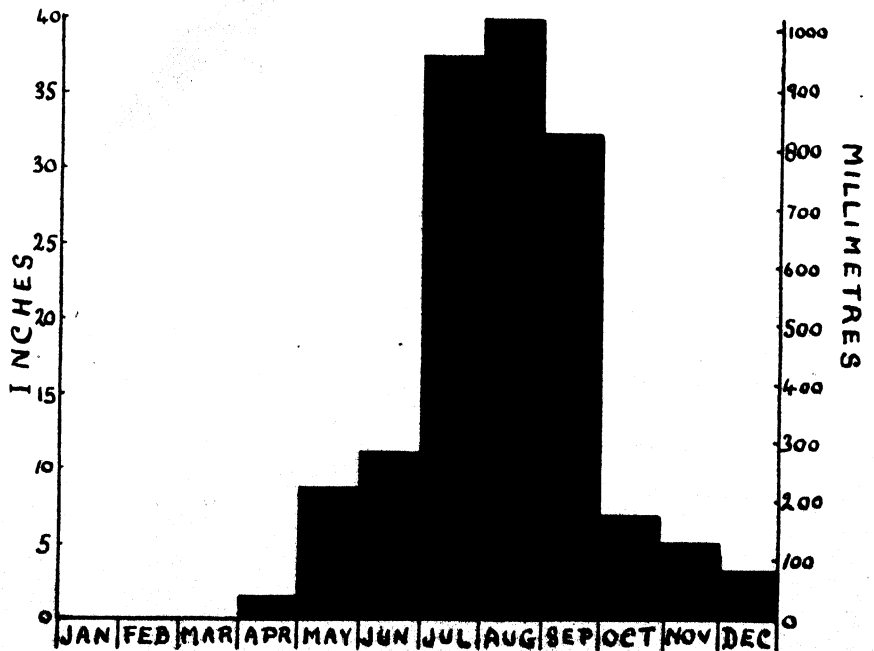
RAINFALL

1943

TOTAL

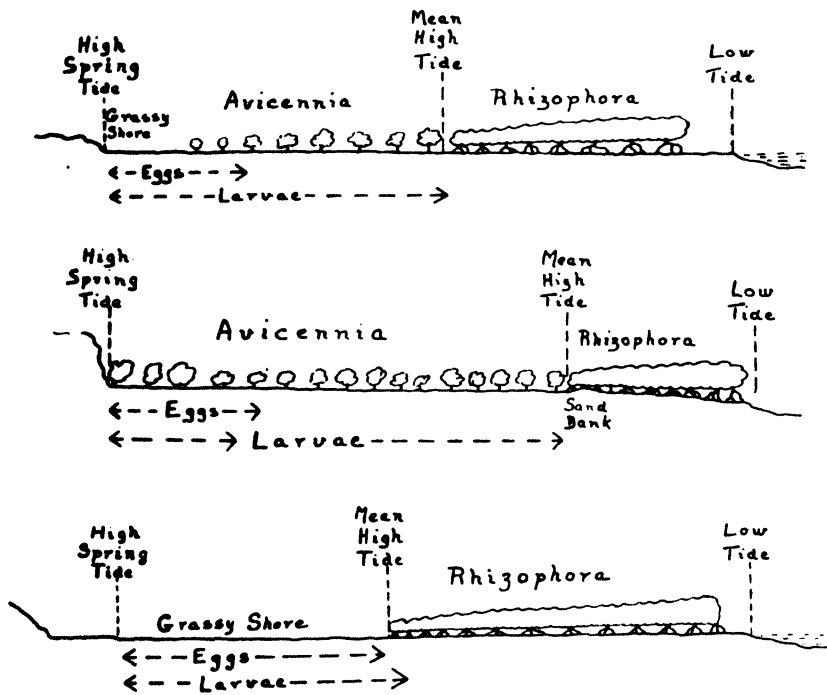
16.9 INCHES

3785 MILLIMETRES





APPENDIX VI, A

DIAGRAMMATIC CROSS-SECTION OF MANGROVE SWAMPDiagrammatic cross-sections of *A. gambiae* var. *melas* breeding-zones.

APPENDIX VI, B

DESCRIPTION OF *Rhizophora* AND *Avicennia* MANGROVE AND THE SEA-GRASS BELT

Two types of mangrove occur, namely, *Rhizophora*, or red mangrove, and *Avicennia*, or black mangrove.

Rhizophora Mangrove. This is the common mangrove which invariably forms the outer zone of the total mangrove area, reaching out into the estuary almost to low-tide level; it may be forest-like in density and height. Approaching the high-tide limit, the height and size of the trees diminish to mere 'scrub' and intermingle with the sea-grass growth. The *Rhizophora* forest floor is a tangled mass of interlacing and branching stilt-like roots; the junction of numbers of these roots to form the trunk of the tree is at or above the level reached by the high tides. The trunks may soar to a height of 100 feet, and the heavy green thick-leaved foliage forms a closed canopy overhead. A characteristic of this mangrove is the flexible drop-roots, which curve out from branches, elongate until they reach soil and water, become embedded, and function as feeding roots.

Avicennia Mangrove. This mangrove occurs in patches or 'orchards' interposed between the sea-grass belt and the *Rhizophora* mangrove; the two types intermingle for a few yards only. The *Avicennia* section of the mangrove area is not flooded by every high tide and frequently is flooded only by high spring tides. In contrast to the tangled growth of *Rhizophora*, black mangrove consists of discrete trees, orchard-like in distribution. The trunk rises direct from the floor of the swamp, and may be up to 18 inches in diameter; an average height is 15-20 feet. The leaves have a different venation from those of *Rhizophora*, and a characteristic feature is the presence on the surface of a crystallized salt exudate. The roots radiate in all directions from the trunk immediately below the surface of the soil; rising vertically from these roots are finger-like processes or pneumatophores; they are densely distributed over the swamp and vary in height to about nine inches; they assist in retarding water-currents and encourage sedimentation, and their decay, together with dead leaves, increases the surface organic debris. The soil is black, peaty and rich in organic content, and in the rain-season may remain a bog for some months; during the dry season it may be dry for the fortnightly or monthly periods between spring tides. Shallow pools are not exposed to the flushing effects of tides, so that in the dry season stagnation and evaporation, and in the wet season dilution by rainfall, cause very considerable variations in the degree of salinity of the water. The inner limits of *Avicennia* mangrove may merge into a sea-grass belt or end abruptly at the foot of low cliffs.

Sea-Grass Zone. The highest tide-level is demarcated by a change in the character of the vegetation: broad-leaved trees, palms and thick undergrowth may give way to a belt of coarse sea-grass, varying in width from a few feet to 150 yards; this sea-grass, or *Paspalum*, has a characteristic appearance, and its density of growth and height are dependent on the type of soil; it is sparse or absent in sandy soils and dense in the rich peaty soils.

(See diagrammatic cross-section of a mangrove swamp shown in Appendix VI, A, and annotated photographs in Appendix VI, C.)



FIG. 1. Close view of sea-grass or *Paspalum*. Aberdeen Creek.



FIG. 3. Sea-grass belt with *Avicennia* mangrove on left and tropical jungle on right. Makanshan.



FIG. 5. Inner edge of *Avicennia* swamp with sea-grass; tropical forest on left. Wellington.



FIG. 2. Sea-grass belt flooded by tide. Aberdeen Creek.



FIG. 4. *Melas* pool in short sea-grass; *Rhizophora* 'scrub' mangrove in background. Aberdeen Creek.



FIG. 6. End of embankment for exclusion of tide; *Avicennia* swamp on right; estuary village in background. Wellington.



FIG. 9. *Rhizophora* mangrove, showing height, stilt- and drop-roots. Bunce River.

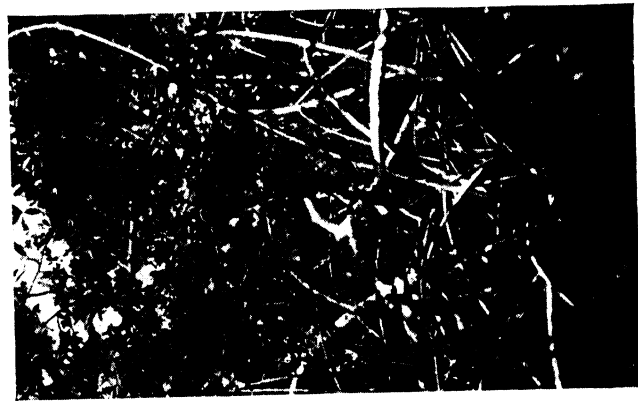


FIG. 10. *Rhizophora* tangle. Makanshan.



FIG. 7. *Avicennia* mangrove. Makanshan.

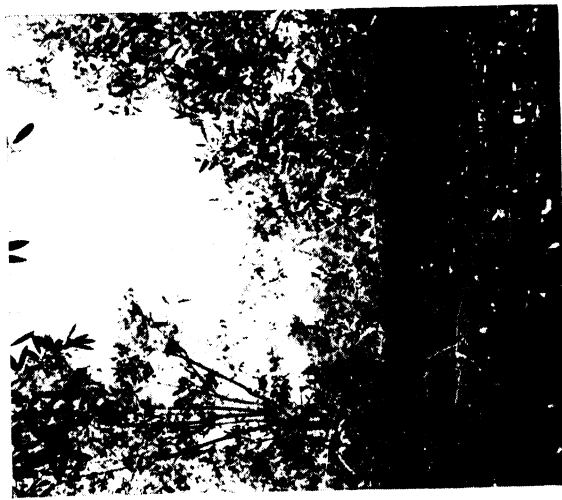


FIG. 8. *Avicennia* swamp, showing pneumatophores. Makanshan.



FIG. 11. *Avicennia* mangrove, showing orchard-like distribution. Wellington.



FIG. 12. *Rhizophora* 'scrub' mangrove. Aberdeen Creek.



FIG. 13. Typical estuary village. Makanshan.



FIG. 14. *Melas* day resting-place in typical African dwelling. Makanshan.

H. R. Grubb, Ltd., Croydon



FIG. 15. Embankment excluding tide, showing perimeter drain of former *melas* breeding-area. Aberdeen Creek.



FIG. 16. As in fig. 15, showing excluded tidal water.



FIG. 17. Limit of high spring tide shown on embankment. Aberdeen Creek.



FIG. 18. Bad photograph of sluiceway with tidal flap. Aberdeen Creek.



FIG. 19. Clearance of *Aicennia* mangrove prior to construction of embankment. Wellington.



FIG. 20. Air-photograph of mangrove belt. Southern shore. Altitude 5,000 feet.

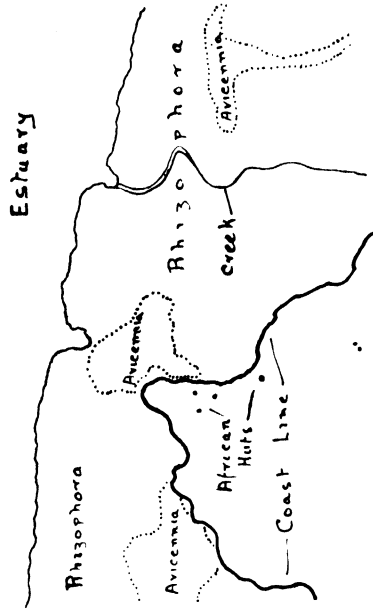
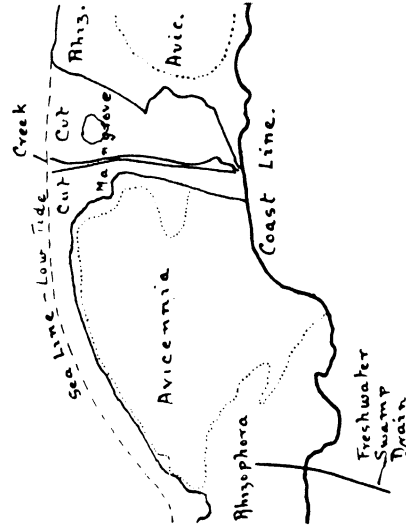


FIG. 21. Air-photograph of *Avicennia* swamp, with adjacent village. Wellington, low tide. Altitude 5,000 feet.



Village

A NEW APPROACH TO TRYPANOSOMIASIS

BY

H. FAIRBAIRN

AND

A. T. CULWICK

WITH A STATISTICAL ANALYSIS BY

F. L. GEE

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(Received for publication May 2nd, 1946)

INTRODUCTION

In 1943 trypanosomiasis in general, and particularly the disease caused by *Trypanosoma rhodesiense*, was discussed by the authors. In the past, trypanosomes, being protozoa, have been regarded rather as fish swimming in a pond, and their reactions to their environment have usually been viewed from this aspect. One of us (A.T.C.), however, thought that they should also be considered as bodies carrying an electrical charge and obeying the laws governing such bodies, and he approached the problem anew from the angle of physical chemistry. His hypothesis and theoretical predictions, and our subsequent experimental work, are the subject of this paper.

PART I

THE HYPOTHESIS

I. THE ELECTRIC CHARGE ON TRYPANOSOMES

In cases of African sleeping sickness, the human blood-stream is invaded by a polymorphic trypanosome which may be regarded as a partially deformable bladder, free to move within certain limits, which we shall consider later, and carrying an electric charge which may be either positive or negative.

T. rhodesiense, *T. gambiense* and also *T. brucei* may occur in the gut of tsetse-flies, either in positively or negatively charged forms, shortly after the fly has fed, but the positive form does not persist. Broom and Brown (1937) have shown that the positive and negative forms are equally infective to the fly, but that a fly fed on a pure strain of positively charged *T. brucei* showed only very few positive forms 48 hours after feeding, while after 72 hours all the trypanosomes present in the gut were negatively charged.

The proventricular forms are negatively charged and, like the gut forms, are long and thin. The salivary-gland forms are short and fat, and have been found to be positively charged under the conditions of the experiments performed.

Rats and humans who contract trypanosomiasis (*T. rhodesiense*) from the bite of an infected fly first show long thin forms in the blood as a rule, and these—at any rate in the rat—are predominantly positively charged, the negative trypanosomes present not exceeding 10 per cent. of the total number. As the infection proceeds, however, the ratio of positive and negative forms fluctuates within wide limits.

Fairbairn, in some unpublished work, has produced a number of graphs showing

the proportion of positive and negative trypanosomes in laboratory animals at different stages of infection by *T. rhodesiense*, the infection being produced in each case by the bite of an infected tsetse-fly, and the sign of the charge determined by the 'salt-concentration' test described by Broom, Brown and Hoare (1936). All these animals showed a predominance of positively charged blood trypanosomes to begin with, but the proportion of negative forms rose rapidly in all cases as the disease progressed, later fluctuating within wide limits. A typical result is shown in fig. 1.

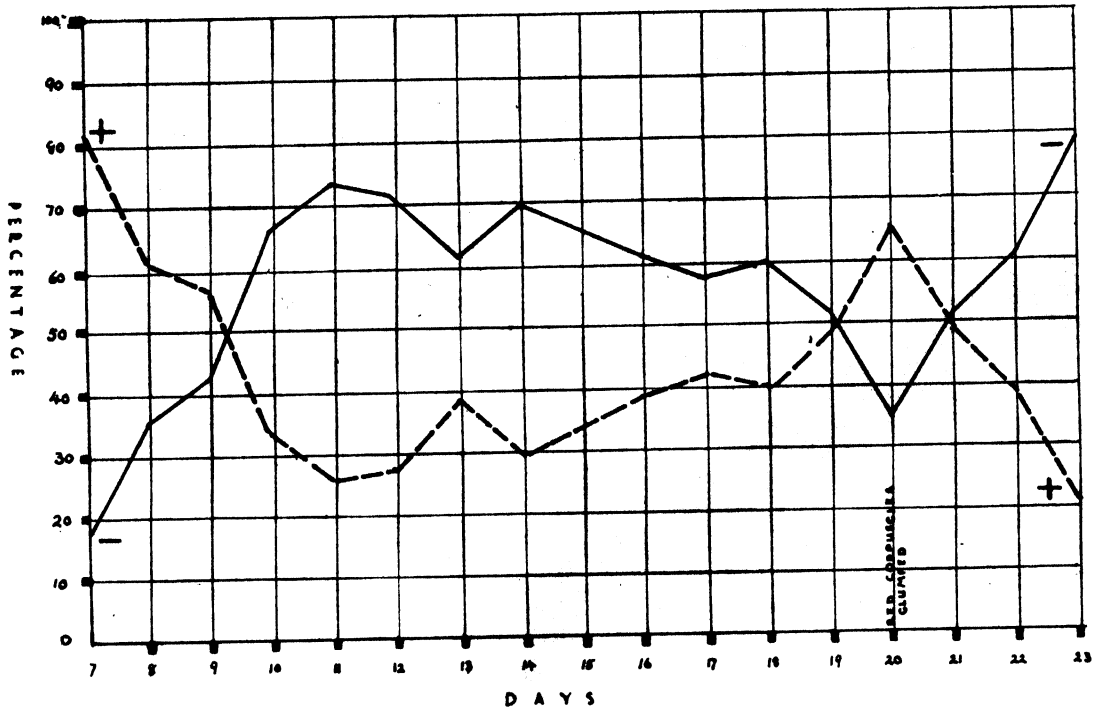


FIG. 1. Graph showing daily proportion of positively and negatively charged trypanosomes (*T. rhodesiense*) in the blood of a rat.

This may be explained if we make the not unreasonable assumption that a trypanosome owes its charge to ionic absorption and/or adsorption, being positively or negatively charged according as to whether it carries an excess of positive or negative ions. This means that the charge on a trypanosome will depend on two factors: first, on the capacity of the flagellate at any time to absorb ions differentially; and, secondly, on the chemical composition of the environment. In other words, the charge on a trypanosome must be considered as an expression of an equilibrium between the organism and its environment.

Now, since the red blood-corpuscles are negatively charged, one would expect them to influence a charged body moving amongst them, provided that the charges involved create an appreciable electrostatic field and are not confined purely to electrical double layers. That such fields* exist in the blood appears highly probable to the author, and we shall therefore consider their theoretical implications.

* Evidence of such fields is given in the experimental section of this paper.

When positively charged salivary-gland trypanosomes are injected into the blood-stream, they will tend to describe paths close to the negatively charged red blood-corpuscles to which they will be attracted. But as soon as their charge changes and they become negatively charged, either as the result of a change in the trypanosome itself or in its environment, they will be repelled by the red cells and will incline to paths remote from the latter.

It follows from this that one would expect the trypanosomes on a thin blood film to be arranged according to a definite rule, and not at random, as hitherto supposed, the positive forms being in close proximity to the red blood-corpuscles, the negative forms being further removed and demonstrating the repulsive forces described above. This might form the basis of a quick and simple technique for determining the sign of the charge on a trypanosome (or for that matter other charged body) in the blood by the examination of a dried preparation.

It also follows that the proportion of positively and negatively charged forms in the blood at any given time will represent the equilibrium point in a reversible process. Where this point is at any particular moment will depend not only on the ability of the trypanosome to change its charge—a property which apparently alters considerably in artificially maintained strains—but also on the composition of the blood. One would therefore expect a constant variation in the ratio of positive and negative forms throughout the disease, and fig. 1 shows that this is the case.

We have seen that in the blood-stream a positively charged trypanosome is subject to electrical attraction, whereas a negatively charged one suffers repulsion; and one would naturally expect a difference in the shapes of the two forms to result, though the degree of difference would naturally depend on the intrinsic capacity of the organism to resist change of shape.

Since the positive form in its path round the corpuscles is constantly subjected to attractive forces, whereas the negative one suffers repulsion from all sides, producing a compression effect, one would expect the negative variant of a trypanosome to be shorter than the corresponding positive variant.

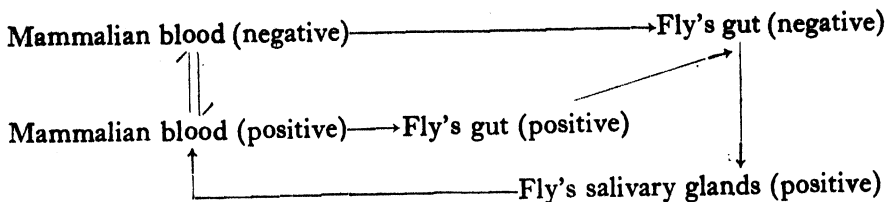
According to this theory, the polymorphism displayed by a trypanosome is, as we have seen, due to at least two factors (*though one must not exclude others*): first, to the property of the organism to hold either a positive or a negative charge; and, secondly, to differences in the electrical and ionic fields in which it moves. It follows from this that a trypanosome removed from the blood and cultured in a homogeneous medium should tend to lose its polymorphism. This does in fact occur.*

As a corollary, one would expect a *truly* monomorphic blood trypanosome—that is, one in which variability (however extensive) can be expressed as a normal Gaussian frequency curve—to be incapable of change of charge.

II. THE EFFECT OF CHARGE ON CYCLICAL TRANSMISSION

We have seen above that the polymorphic trypanosomes appear to undergo several changes of charge when cyclically transmitted through the tsetse-fly. They are:

* The experimental work described later shows that this is only one reason, and not the most important one, for such monomorphism.



and it would appear that this ability to change charge is essential to the cyclical transmission of the disease in nature, since positive forms cannot persist in the gut of the fly, whereas negative forms are not found in the salivary glands. I have so far been unable to find any instance of a trypanosome which can only carry a charge of one sign being able to establish itself in both the gut and the salivary glands of any insect; it is, indeed, very doubtful whether such an instance will ever be forthcoming, since ability to exist and develop in such chemically diverse environments naturally involves a corresponding ability on the part of the organism to adjust itself to very different ionic effects.

Murgatroyd and Yorke (1937), analysing the gradual alteration of a strain of *T. brucei* on syringe passage, stated that in guinea-pigs, so long as the strain remained transmissible by *Glossina*, its original characters appeared to be preserved practically unchanged. Lengthy syringe passage, however, alters many of the properties of a strain, and may impair the capacity for changing charge so seriously that the strain is no longer transmissible by the tsetse-fly. Broom, Brown and Hoare (1936) describe such a strain of *T. brucei* which during 'four generations in mice . . . invariably remained positively charged,' the first two authors later reporting in 1937 and again in 1939 that this strain had lost its transmissibility by the fly.

III. CHANGE OF CHARGE ON RELAPSE AND ANTIBODY FORMATION

The normal infection in nature, i.e., from the tsetse-fly, consists of positively charged salivary-gland forms which first produce positive forms in the blood, though after a time changes of charge occur, and a proportion of negatively charged forms are produced. In such an infection the body of the host is called upon to produce antibodies capable of acting on both the positive and the negative variants.

If, on the other hand, an animal is inoculated with a 'fixed' strain, which produces either positive or negative trypanosomes but not both together at any one time, only one antibody will be produced in the blood, and if the antibody is strong enough the infection will die out—that is, always supposing that the strain is truly 'fixed.'

Say, for the sake of argument, that the original strain was positively charged. The positive trypanosomes as they increase will produce a rising concentration of the homologous antibody, which will, if strong enough, eliminate them. In a strain which is only partially 'fixed,' however, a few negative trypanosomes will be formed and left behind, unaffected as they are by the positive antibody (Broom and Brown, 1940), to produce a relapse, after an apparently spontaneous cure, in which the trypanosomes will be negatively charged. Similarly, an infection with the negative variant will produce positive trypanosomes on relapse. This change of charge on relapse has been described by Broom, Brown and Hoare (1936).

If, however, either positive or negative trypanosomes from this relapsing animal are subinoculated into another, the great bulk of them should remain positively or negatively charged respectively until the formation of antibodies, coupled with relapse, brings about

a reversal of charge. This is in accordance with the observed facts. Broom, Brown and Hoare (1936) have shown that 'No alteration in the sign of the charge takes place in continuous progressive infections during serial passage [i.e., syringe passage] in mice for more than thirty generations,' but that 'the sign of the charge of the trypanosomes which reappear after apparent spontaneous cure and subsequent relapse in mice is invariably the opposite of that carried by the original strain.'

It seems curious at first sight that spontaneous cure should not take place in a normal (fly-borne) infection in which the trypanosomes occur as mixed positive and negative variants in the blood. One would expect both the homologous antibodies to be formed, with a subsequent dying-out of the infection in at least an appreciable number of cases; but this is not so. It seems that the probable answer to this problem is that the antibodies themselves carry charges, and therefore tend to neutralize one another and also to affect the charges on other bodies in the blood. Referring to fig. 1, it is noteworthy that in the great majority of these cases, of which the graph reproduced represents but one, after the infection had been established for some days a rapid drop in the proportion of negative trypanosomes was marked by a clumping of the red blood-corpuscles. This may be explained by supposing that the concentration of the antibody affecting the negative trypanosomes rises as the number of negative trypanosomes do, until a concentration is reached when it begins to affect the negatively charged red cells too. This would account for the observed clumping just after a rapid drop in the proportion of the negative trypanosomes had occurred. In no case did a rapid diminution of the positive forms produce this result. The auto-agglutination of the blood in human sleeping sickness cases is possibly caused in the same way.

One would therefore expect infections with positively and negatively charged trypanosomes to produce different effects. The positive antibody should tend to stabilize, rather than to upset, the electrical charges of the red blood-corpuscles, and therefore one would expect that the body's resistance to positively charged forms would be far greater under normal conditions than to negatively charged ones of the same strain. Therefore, the infectivity of a given strain of trypanosomes to man would, other things being equal, depend on the rate at which it could produce negative blood forms, because the longer it persisted in pure, or predominantly, positive form, the greater would be the chance of the infection dying out as the result of the rising concentration of the homologous antibody, and this is not limited, as in the case of the negative antibody, by the effects which the latter has on the charges of the red cells. If, then, the rate of production of negative blood forms could be diminished by passages through the rat, as seems to occur on syringe passage, then one would expect the early blood forms from the rat to be less infective to man than the 'electrically more versatile' metacyclic forms from the fly.

The possibility that the antibodies affecting the positively and the negatively charged forms react with one another is one which should not be ignored in considering possible immunizing techniques; because, if this interaction does occur, it is obviously useless to produce both together in the blood. This may account for the general lack of success which has attended attempts at producing an active immunity based on antigens derived from mixtures of positively and negatively charged trypanosomes.

IV. RED-CELL ADHESION AND SERUM REACTIONS

It has been shown that, if infected blood is suspended in isotonic glucose solution,

positively charged trypanosomes adhere to the negatively charged red blood-cells, while the negatively charged trypanosomes remain free, as one would expect on our theory (Szent-Györgyi, 1921).

Broom, Brown and Hoare (1936), using 'both the adhesion test of Davis and Brown (1927) and the protection test described by Schilling and Neumann (1932),' found that, 'In the case of *T. evansi*, a serum prepared against the positive variant' caused '100 per cent. adherence with the homologous trypanosome but gave no adhesion with the negatively charged relapse variant. On the other hand a serum prepared against the negative variant caused 100 per cent. adhesion with both variants.' This is, according to our theory, to be expected. Adhesion with the positive variant involves the breaking down of the 'protective' action of whatever substance (or substances) inhibits adhesion. Adhesion of the negative variant involves this too, but, over and above, it necessitates the suppression of the mutual electrical repulsion of the corpuscles and the negatively charged trypanosomes. In other words, the 'negative serum' has to do all that the 'positive serum' does and more, and so it is to be expected that the 'negative serum' would cause adhesion in the case of both the positive and negative variants.

Cyclical transmission through the tsetse-fly necessitates a change of charge in the case of those trypanosomes which exist and develop in both the gut and the salivary glands, and it is not therefore surprising that many of the old 'fixed' strains are incapable of undergoing normal cyclical transmission.

It follows from this that, if one could succeed in infecting a tsetse with an old strain of a trypanosome which had not wholly lost its power of changing its charge, selection should occur in the fly, only those trypanosomes surviving which had retained most of their original properties. In other words, the passage of positive and negative substrains through the tsetse should by a process of selection produce a strain of mixed positive and negative blood forms closely resembling the original strain from which the substrains were derived. Therefore, one would expect that an animal infected by a fly fed on either a positive or a negative variant of an old strain would develop a normal infection in which both positive and negative trypanosomes appeared side by side. One would also expect that cyclical substrains produced by the passage of positive and negative variants of an original strain through the tsetse would resemble each other closely in serological type, irrespective of which variant the flies were fed on. This has been found to be the case (Broom and Brown, 1940).

It may be wondered why positive trypanosomes do not normally adhere to negatively charged red cells in the blood. Szent-Györgyi (1921) contended that the serum proteins had a 'protective' action, and, as mentioned above, he overcame this possible protection by suspending infected blood in isotonic glucose solution to precipitate the globulins, and found that adhesion took place.

Broom, Brown and Hoare (1936) consider that, 'If this is the true mechanism, removal of proteins by washing should allow adhesion to take place when red cells and trypanosomes are re-suspended in saline,' and find that such is not the case, even after repeated washing in a centrifuge.

This argument, however, seems unsound. The protective action is most probably due to surface-tension effects produced on the trypanosome-liquid and corpuscle-liquid interfaces in layers which are but a few molecules thick, and whose adherence to these bodies is far too strong to be broken by any centrifuge, but whose action can be overcome

by means which alter the surface-tension effects. Broom, Brown and Hoare's experiment therefore does not disprove Szent-Györgyi's contention, as his chemical means of precipitating the globulins would be expected to be far more efficacious than the former workers' mechanical method.

V. SUSCEPTIBILITY TO ARSENICALS

Broom, Brown and Hoare (1936) have shown 'that the positively charged variant [of a trypanosome] is distinctly more susceptible to the action of the negatively charged ion of tryparsamide,' as one might expect. But the negative variant is susceptible too, only to larger doses.

If the greater susceptibility of the positive variant is due to the natural attraction of a positively charged body to negative ions, one might ask why the negative trypanosome is affected at all. Such a question, however, implies the idea of a static ionic condition in the blood plasma, whereas in point of fact the ions are moving rapidly in random motion, subjecting all bodies in the plasma to constant bombardment.

The negative ions of tryparsamide will naturally tend to be repelled by a negatively charged trypanosome, but those moving fast enough towards it will reach the organism, the number doing so in a given time depending, of course, on the concentration of the drug in the blood, so that one would expect that, once a certain concentration had been reached, the negative variant would succumb, as is the case.

VI. ADAPTABILITY AND INHERITANCE

The polymorphic trypanosomes are highly adaptable organisms. In their natural state they can change their charge and their shape. By prolonged syringe passage they can form a number of variants possessing different electrical, morphological, chemical and pathological properties. Such passages invariably tend to a fixation of type.

With the exception of some drug-fast strains (e.g., tryparsamide), normal cyclical transmission through the insect vector, where this is still possible, reproduces a strain very similar in all properties to the original strain from which the aberrant strains were derived. In certain cases even drug-resistance may completely disappear on cyclical transmission, as in the case of the Gemena Bayer-resistant strain of *T. gambiense* passed through *Glossina palpalis* (van Hoof, Henrard and Peel, 1938).

Passage through mammalian hosts may also profoundly influence the properties of a strain. Frequently considerable difficulty is experienced in establishing a naturally occurring strain in laboratory animals; yet, after the conclusion of a few successful passages—perhaps only one—no trouble occurs with subsequent inoculations. In this respect it is interesting that Bruce (1915) describes three strains of '*T. brucei* vel *rhodesiense*,' isolated from naturally infected dogs in Nyasaland, to which both monkeys and guinea-pigs were extremely resistant—two animals which are, of course, regularly used for passing this trypanosome under laboratory conditions.

It is frequently most difficult to infect rats with *T. gambiense* from humans, but, if it is first passed through a monkey, no trouble is experienced.

Corson (1934) describes a strain of *T. brucei* from a naturally infected monkey, which was passed through a dog by syringe, and then inoculated into rats, and states that its virulence to the rat was found to be 'unusually low,' that it failed 'to infect, in the earlier passages, about half of the rats inoculated,' and that it 'produced a latent infection in

one rat for about nine months.' Corson then goes on to describe how a substrain derived from this last animal killed rats in from 13 to 19 days—an 'increased virulence maintained in subsequent direct passages'—and how this substrain also infected guinea-pigs which had, as we have seen, resisted the original strain.

These changes, and many others which trypanosomes can undergo, are no mere ephemeral variations in the properties of a strain; on the contrary, they partake of the nature of genetic segregation, involving, as they do, the alteration of heritable characters. In the writer's opinion, it is impossible adequately to explain them all without assuming that trypanosomes undergo syngamy, which produces a heterogeneous population, composed of individuals differing in their genetic composition, whose nature is then moulded by a process of selection governed by environmental influences. This view seems to be supported by the work of Lloyd (1930), who showed that, by mixing two old strains of *T. brucei* in a single host, he obtained greater transmissibility by *Glossina* than from either strain alone.

VII. CONCLUSION

It will be seen that much that is known regarding the polymorphic trypanosomes can be explained if we assume that the electrical charges which they carry greatly influence their behaviour, that they obey the laws governing charged bodies, and that their properties are maintained in nature by syngamy, which is associated with cyclical transmission, but undergo considerable modification on prolonged syringe passage, producing aberrant strains on which it is dangerous to base conclusions regarding the naturally occurring organism.

PART II EXPERIMENTAL WORK

VIII. THE EFFECT OF THE SURFACE CHARGES ON RED BLOOD-CORPUSCLES

A critic of the above hypothesis called in question the assumption that the surface charge of a red blood-corpuscle had an appreciable effect on another charged body at a distance. He maintained that the electric field round a red cell probably fell off extremely quickly as one passed away from its surface, and would appear to become negligible at a distance too small to affect a trypanosome.

We accordingly made camera-lucida drawings of isolated pairs of red blood-corpuscles on a stained thin film, selecting only those which lay perfectly flat and which were deformed symmetrically.

By measuring the circumference of two corpuscles, A and B (fig. 2), their diameters in the undeformed state were calculated, from which we computed the degree of deformation of each, y_a and y_b . The undeformed corpuscles are shown as two circles with centres O_a and O_b .

The distance, d_a , and the corresponding one for the other corpuscle, d_b , were then found. Taking the average deformation and distance for each pair of corpuscles examined, we obtained two functions, y and d , where

$$y = \frac{y_a + y_b}{2} \quad \text{and}$$

$$d = \frac{d_a + d_b}{2}$$

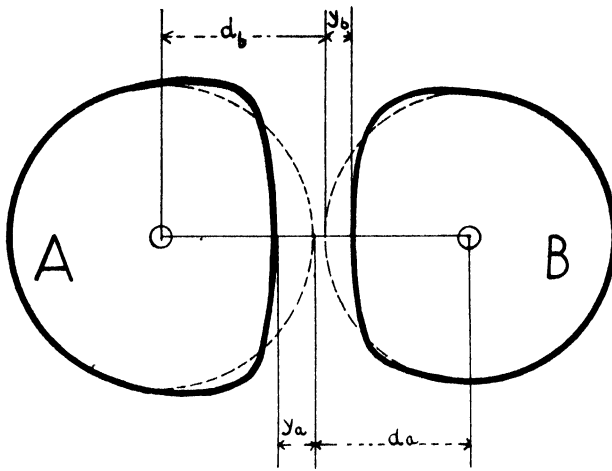


FIG. 2

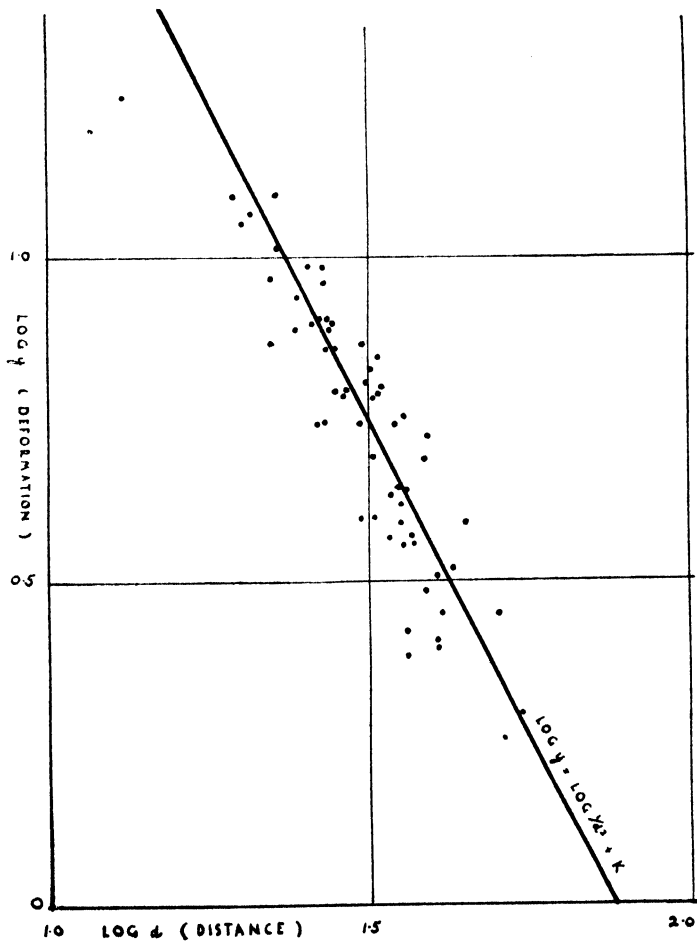


FIG. 3

Log y was then plotted against log d , and the result is shown in fig. 3. It will be seen that, within the limits of experimental error, the points were distributed over a wide range of values along a line whose equation was $\log y = \log \frac{1}{d^2} + K$ (a constant), indicating that the deformation, y , varied inversely as the square of the distance, d . Statistical analysis showed that, ignoring the two points in the left-hand top corner of fig. 3, there was no significant departure from a straight line.

The two points referred to have their parallel in the deformation curve of any elastic body, and their departure from the straight line indicates that in a red corpuscle, as in any other elastic body, a limiting value is reached after which the constancy of the stress/strain relationship no longer holds good.

Observations carried out on fresh films, and measurements from them, indicate that these results could not be attributed to the drying of the stained films. The main group of figures was obtained from stained films, rather than from fresh ones, because the former made possible the use of a higher degree of magnification and rendered the measurement of deformation a more accurate proceeding.

This experiment indicates that red blood-corpuscles mutually repel one another, and that the repelling force follows the inverse square law. The deformation of red blood-corpuscles can be seen well in Plate XVII. We therefore conclude that, over and above any purely double-layer effects exhibited, the red blood-corpuscle possesses a charge producing an electrostatic field the effects of which are appreciable at the distances with which we are here concerned.

Similar cases have been noted in which trypanosomes, presumably negatively charged, have deformed red blood-corpuscles by approaching them, but without actual contact (see fig. 4, A, and Plate XVII, figs. 19 and 21). This too suggests electrical action at a distance.

It is appreciated that the existence of an electrostatic field of such dimensions round a cell cannot be attributed to a ζ potential, but implies that the cell is capable of maintaining a relationship with its environment which does not conform to the usually accepted views on diffusion equilibria.

A further series of experiments, carried out on defibrinated blood, gave the following results. The corpuscles in a recently drawn sample showed the mutual repulsion described above; but ageing for six hours destroyed this effect, while the addition of one drop of 40 per cent. formaldehyde solution to 1 c.cm. of blood did the same in 30 minutes, but not in 10.

We therefore suggest that this property of maintaining an electrostatic field round the cell, and the relationship between the latter and its environment which this involves, is associated with the living nature of the protoplasm and the cell's biological activity, and is destroyed when this is extinguished.

The whole question obviously has most far-reaching implications. Its further study deserves attention, since it may well throw light on the little-understood energy exchanges and transformations in living tissue; and it is therefore to be hoped that those with the appropriate facilities at their command will carry the investigation further.

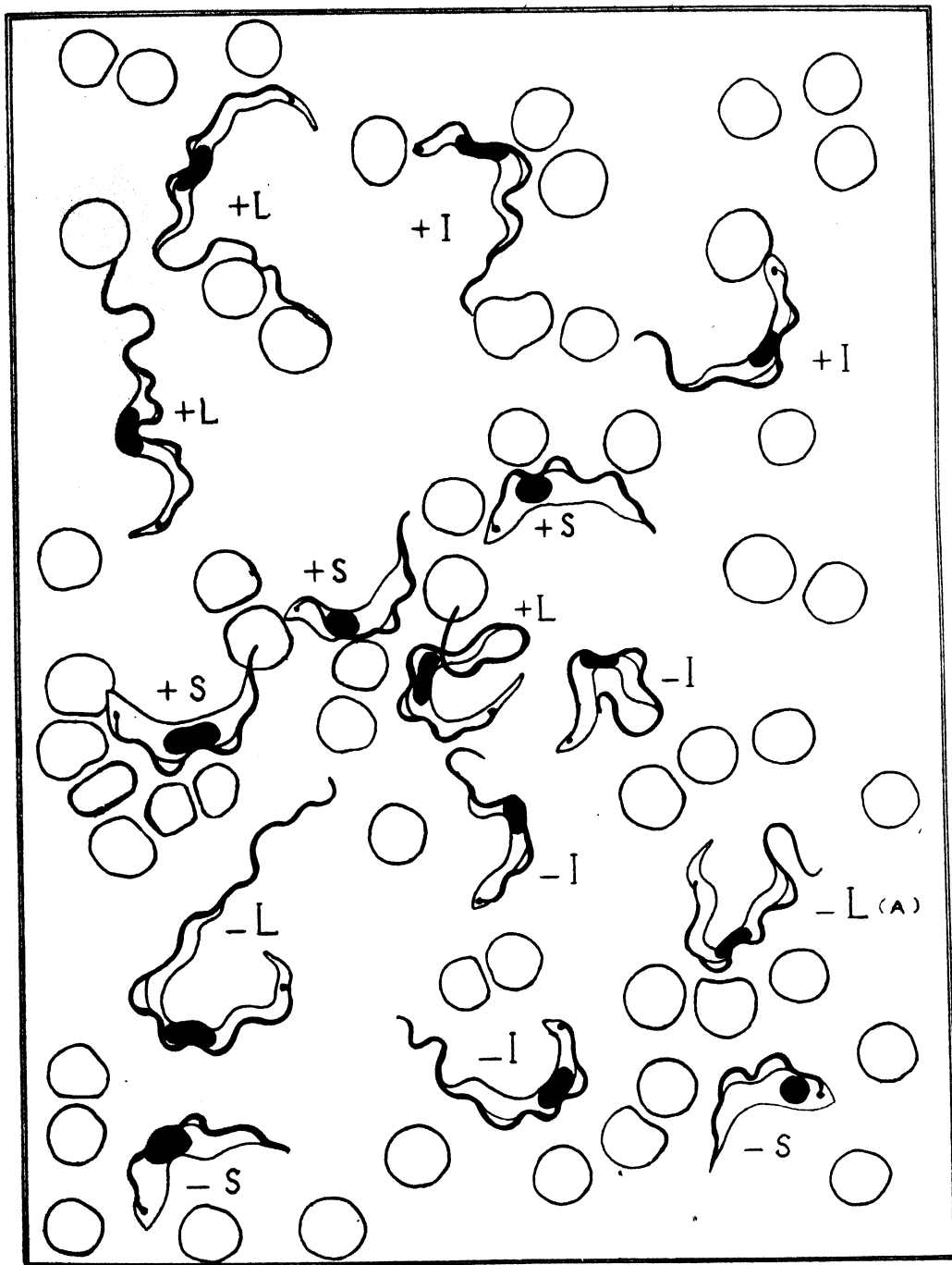


FIG. 4. The relationship of positively and negatively charged forms of *T. rhodesiense* to the red blood corpuscles in a thin blood film.

+. Positively charged trypanosome.
L. Long form.

-. Negatively charged trypanosome.
I. Intermediate form.
S. Short form.

IX. THE DETERMINATION OF THE ELECTRICAL CHARGE ON THE TRYPANOSOME

When the hypothesis was completed in February, 1944, an examination of stained films, made from the blood of rats at different stages of their infection, showed that apparently the trypanosomes were not distributed at random, but exhibited the predicted effects of attraction to, or repulsion from, the negatively charged red blood-corpuscles. Fig. 4 is a camera-lucida drawing of a typical field.

To confirm this, a series of parallel experiments* was done in which the percentage of positively to negatively charged trypanosomes in the blood of a number of rats was determined at the same time (1) by examining an ordinary stained thin blood film, and (2) by Broom, Brown and Hoare's technique (1936), using their 10 per cent. normal saline in 4 per cent. glucose solution. The results of the two methods are in close agreement, and are shown in Table I.

TABLE I

Showing the percentage of positively charged trypanosomes in the blood of rats infected with *T. rhodesiense*, as determined by (a) the salt-concentration test and (b) the thin-film method

Rat no.	By salt-concentration test	By thin-film method
7767	90	90
7782	93	94
7639	52	55
7641	62	63
7645	66	67
7693	50	49
7693a	53	48
7693b	53	55
7766	49	51
7776	83	79
7761	90	91
7760	69	67
7766	55	50

Broom, Brown and Hoare (1936) have shown that their salt-concentration test gave results corresponding very closely with those obtained by directly determining the sign of a trypanosome's charge in a cataphoresis cell. Since the results of the thin-film method correspond so closely with those obtained in the salt-concentration test, we concluded that the trypanosomes were not distributed at random, and that the sign of the charge could be determined accurately by the examination of a thin film.† This method was therefore used in all the subsequent work.

X. THE VARIOUS TYPES OF *T. rhodesiense* AND THEIR DEVELOPMENT

Muriel Robertson's (1913a) definition of the short form of *T. gambiense* was those up to 20μ in length, but this was a purely arbitrary criterion. Vanderplank (1944) stated that *T. rhodesiense* had two chromosome types; and in a personal communication he informed us that the trypanosomes with six chromosomes were long and thin, usually

* All the experiments described below were carried out with the strain of *T. rhodesiense* maintained in this laboratory and transmitted cyclically by laboratory-bred *G. morsitans*.

† The slides used must be absolutely free from grease, and the films must be very thin, with the corpuscles well spaced and uncrenellated.

with a long free flagellum, while those with five chromosomes were short and stout, with no flagellum or with one usually less than 10 per cent. of the total body length. We adopted his morphological classification in the experiments described below, though subsequent work (see Section XIII) showed that his views on the chromosome structure of the short form were untenable.

When men, monkeys and rats were experimentally infected by the bite of infected tsetse-flies, it had been found that the earliest trypanosome to appear in the blood (and in the arm-reaction of men) was usually the long, thin, positively charged form. In order to determine the speed of development of the other forms, and to find out when the negatively charged variants appeared, rat 6503 was infected by the bite of fly OL. 15 ex Thomson's gazelle 66, and from the second day of its blood being microscopically positive a thin film was made daily for 15 successive days, the trypanosomes, chosen at random, being measured and their charges determined. A total of 8,794 trypanosomes was measured in the 15 slides. It was found that under 16μ all the trypanosomes were stout, from 17μ to 21μ the majority were stout, between 22μ and 26μ the majority were thin, while all the trypanosomes of 27μ and over were thin.

The daily percentage of the various forms present, divided into stout and thin, positive and negative, with the fluctuation in their charges is shown in Table II.

TABLE II
The percentages of the various forms of trypanosomes found on 15 consecutive daily slides

Day	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th
Stout, negative	0.2	4.1	6.4	7.1	15.0	15.7	16.0	12.2	16.2	12.1	21.8	16.1	22.2	11.5	4.1
" positive	1.8	9.0	12.4	23.3	36.1	35.8	26.0	22.9	29.7	25.4	12.3	22.8	11.4	8.8	6.6
Thin, negative	4.1	10.0	17.0	7.1	9.4	7.4	9.5	13.9	10.1	6.5	3.3	9.5	10.0	3.6	22.0
" positive	93.9	77.0	64.1	62.3	39.4	41.0	48.4	50.9	43.9	56.0	62.6	51.5	56.3	76.0	67.2
No. of trypano- somes measured	461	535	614	627	626	578	588	589	572	583	455	652	559	824	531

It will be noticed that the trypanosomes were predominantly positively charged during the early stages of the infection, but that the proportion of negatively charged flagellates rose rapidly during the first few days, after which it fluctuated. The result was of the same type as that shown in fig. 1, where the charge had been determined by Broom, Brown and Hoare's technique.

The length-distribution curve for *T. rhodesiense* is given in fig. 5. It will be seen that, allowing for the difference in scale, the curve for all forms combined bears a close resemblance to that for *T. brucei* (Bruce, 1911; reproduced by Wenyon, 1926). It does not represent a statistically normal distribution. When, however, the trypanosomes were divided on our classification into stout and thin, positive or negative forms, then an entirely different picture was obtained. It was found that the distribution of the lengths could now be divided into four distinct curves, one for each of the four types, viz., stout positive and negative, and thin positive and negative (fig. 5).

These results were submitted to Mr. F. L. Gee, of Makerere College, Uganda, for statistical analysis, and his report, with the length-distribution figures, is attached as an Appendix. He has produced powerful evidence that this strain of *T. rhodesiense* consists

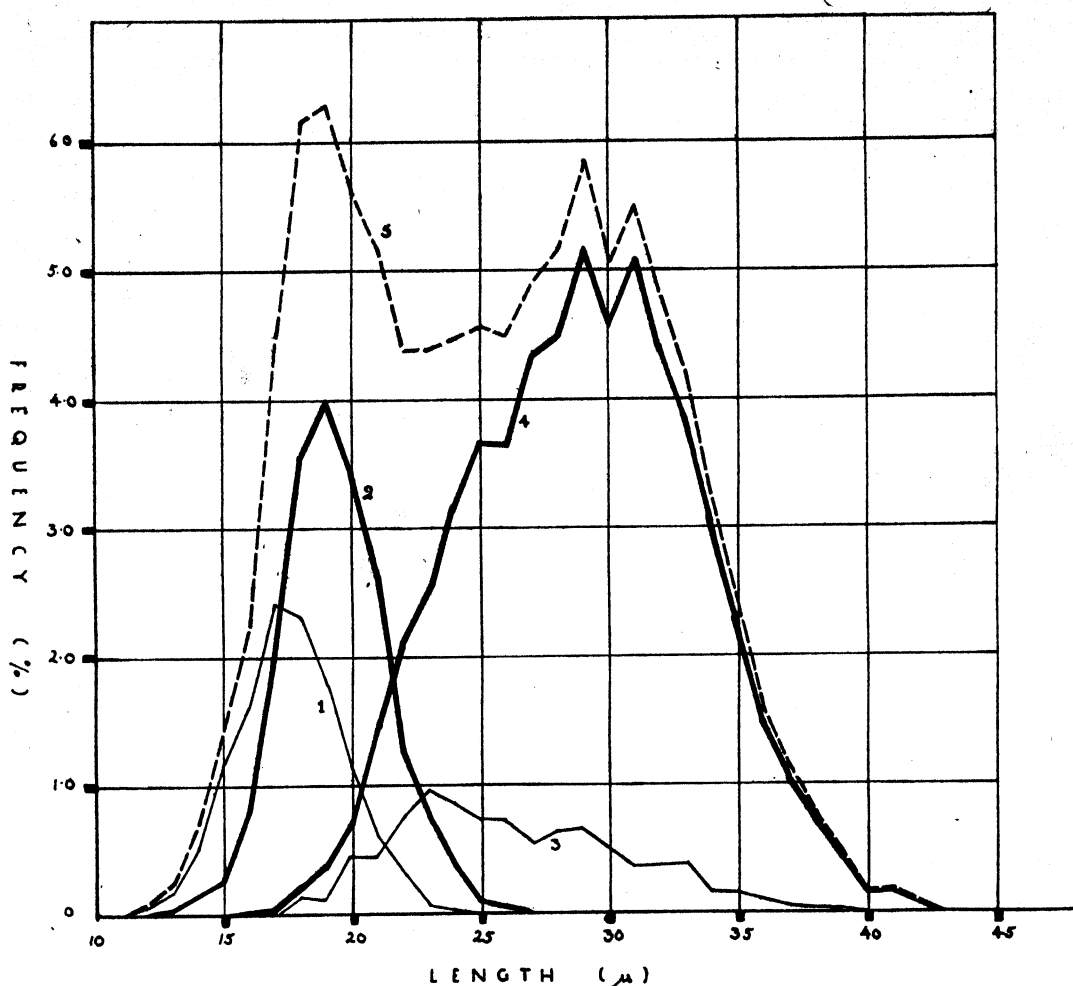


FIG. 5. The length distribution of *T. rhodesiense* in rat 6503.

- | | |
|-------------------------|-------------------------|
| 1. Stout negative form. | 2. Stout positive form. |
| 3. Thin negative form. | 4. Thin positive form. |
| 5. All forms combined. | |

of three main populations, each occurring as two electrical variants, positively or negatively charged, making six types in all. The mean lengths were as follows:

Form	No. of trypanosomes measured	Mean length
Stout negative	1,067	17.555 μ
" positive	1,694	19.320 μ
Thin negative	838	22.65 μ and 28.24 μ
" positive	5,195	23.897 μ and 30.380 μ
Total ...	8,794	

The final resolution of the length distribution into six Gaussian curves is shown in fig. 6.* The results confirm both the validity of the classification used and the method of determining the charge; and it will be seen that in each case the mean length of the positively charged variant is significantly longer than that of the corresponding negative one, as was predicted in the section above entitled 'The Hypothesis.'

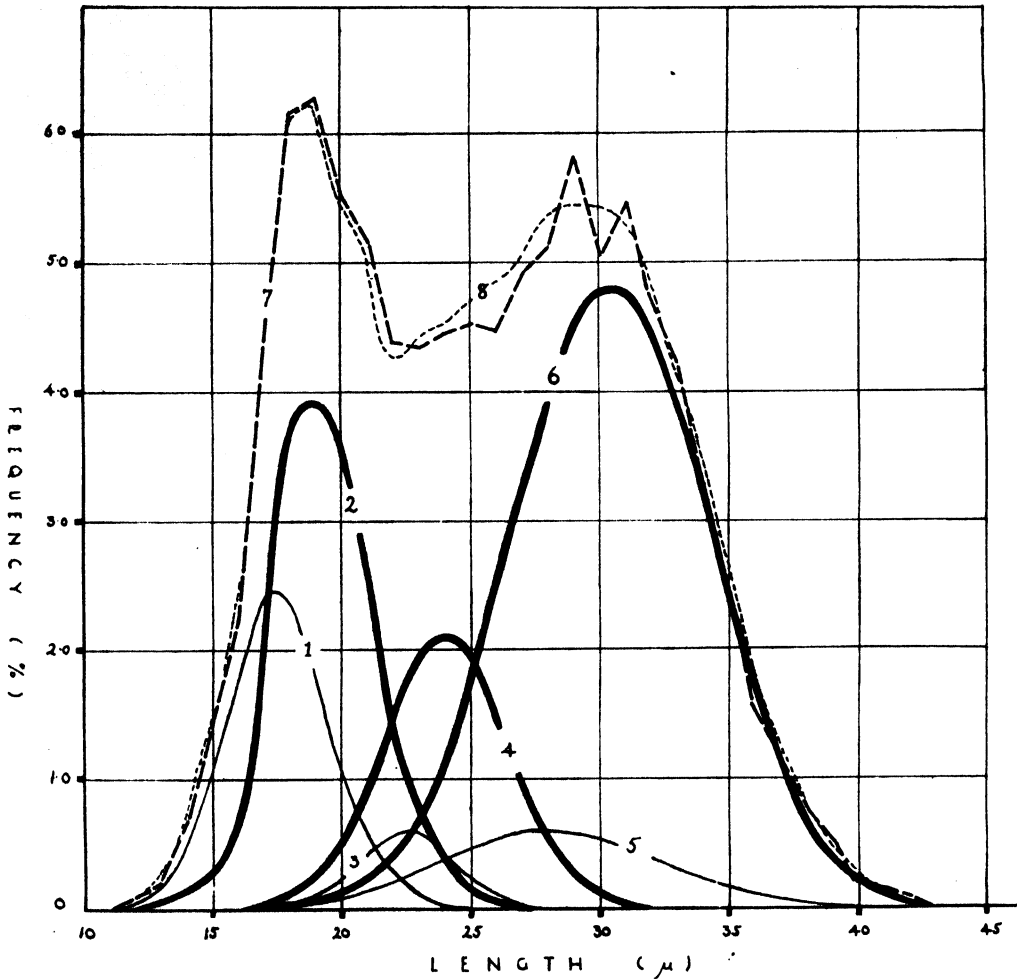


FIG. 6. The final resolution of *T. rhodesiense* into six types.

- | | |
|--------------------------------|--|
| 1. Short negative form. | 2. Short positive form. |
| 3. Intermediate negative form. | 4. Intermediate positive form. |
| 5. Long negative form. | 6. Long positive form. |
| 7. Total as found. | 8. Total as calculated from sum of curves 1-6. |

Reference to the Appendix and to fig. 6 shows that the positively charged, long, thin form with a mean length of 30.380μ was the dominant one, constituting over 46 per cent. of the total.

* The fact that the lengths of *T. evansi* in horse and camel recorded by Bruce (1912) and for *T. vivax* in ox (Bruce *et al.*, 1911) are not normally distributed ($P < .01$) suggests that the so-called 'monomorphism' of these trypanosomes requires further investigation.

During the examination of the above films, Mrs. R. E. Culwick pointed out that the thin trypanosomes could be divided into two morphological groups. In one group the trypanosomes had long narrow posterior extremities, with the kinetoplast some considerable distance—perhaps as much as 4μ —from the posterior end, while in the other group the trypanosomes had a much blunter posterior end. The two forms are illustrated in fig. 4. When the positively and negatively charged variants of these two types were measured, each produced a normal Gaussian distribution curve. The mean lengths, which were all significantly different from one another, were (negative) $22.38\mu \pm 0.14\mu$ and $28.67\mu \pm 0.13\mu$, (positive) $23.45\mu \pm 0.19\mu$ and $29.68\mu \pm 0.18\mu$. The close correspondence between these figures and Gee's is striking when one considers the totally different methods by which the two sets were obtained.

It therefore seems to be established that the long trypanosome with the narrow posterior end and with the kinetoplast some distance anterior is the true long form, while the other blunter-ended trypanosome is the intermediate form, as isolated by Gee's statistical analysis. It will be noticed that once again the mean length of the positive variant is significantly greater than that of the corresponding negatively charged one.

XI. THE POSTERIOR-NUCLEAR FORM

It was found that, if a drop of infected rat's blood on a slide was placed in a saturated aqueous atmosphere for five minutes and was then smeared, the proportion of posterior-nuclear forms of *T. rhodesiense* was far greater than in a control-slide in which the blood had been smeared immediately it had been withdrawn.

Posterior-nuclear forms are supposed to occur only when *T. rhodesiense* is inoculated into the smaller laboratory animals. Plate XVIII, figs. 28 and 29, however, are photomicrographs of posterior-nuclear forms of *T. rhodesiense* in the blood of a European male*, and fig. 30 gives an idea of the heavy infection from which he was suffering.

XII. SYNGAMY

Vanderplank (1944) has postulated a meiotic division in trypanosomes, by which gametes are extruded, and he thought that these gametes united with the reduced nucleus of another trypanosome. On the other hand, in the slide made on the second day from rat 6503 (*supra*) were two trypanosomes which appeared to be fused in a 'head-to-tail' position, with their nuclei in juxtaposition, which suggested syngamy of another type.

To study the process *in vitro*, a trypanosome suspension was made in Ringer-glucose† solution, and the trypanosomes were examined in a 0.1 mm.-deep cell for periods of up to two hours in duration. The cell used had an open side, and the fluid under the cover-slip was in contact with a large drop left uncovered on the slide, so that atmospheric oxygen could diffuse through the solution. Distilled water was added as required to the drop on the slide to counteract evaporation. The tube containing the suspension was shaken frequently to keep it aerated; and, if the trypanosomes in the cell lost their motility, the

* We are indebted to Dr. Gabathuler, of Mahenge, under whose care this patient was, for the slides from which the photomicrographs were made.

† Ringer-glucose is the solution evolved by Yorke, Adams and Murgatroyd (1930).

preparation was put up again from the suspension in the tube, which was of the same age. A half-inch objective and a $\times 20$ eyepiece were used, which gave reasonable magnification combined with depth of focus.

Usually within 20 minutes of starting an experiment—though on one occasion we had to wait nearly two hours—the following sequence of events took place. Two trypanosomes approached one another and intertwined, their centres in contact, their anterior and posterior ends free. After a short period the anterior end of each fused with the posterior end of the other, producing a form as in Plate XV, fig. 5.

The fusiform body then elongated very considerably, and the serpentine motions of its two halves appeared to be unco-ordinated, often giving the appearance of one half tugging against the other. This form was comparatively stable, often persisting under these conditions for longer than one could keep the preparation motile on the slide. Castellani (1903, Plate II, fig. 27) has drawn such a 'conjugation form,' found in the blood of a case of sleeping sickness, and Muriel Robertson (1913c, Plate V, fig. 39a) also figured a similar form of *T. gambiense* found alive in the gut of *G. palpalis* three days after the ingestion of trypanosomes.

After a period of time, which might be short or long, the fusiform body divided at its centre, producing two trypanosomes. Sometimes, however, one of the trypanosomes appeared to degenerate before the fusiform body divided, forming a protoplasmic mass to which the other trypanosome was attached by its posterior end and from which it might, or might not, free itself. In one instance we kept a trypanosome, which had freed itself, under continual observation, and saw it divide normally later. In those cases where the trypanosome did not break free, it too lost its trypanosome shape, curled up, and fused with the mass which was the other trypanosome, becoming inert.

Several instances were seen of more than two trypanosomes fusing together. A highly motile trypanosome would encounter a fusiform body and would attach itself to it, forming a body with three flagella. The stability of such bodies varied greatly. Sometimes the third trypanosome became detached after only a few seconds; sometimes it remained adherent for a very long time and was never seen to break away.

The forms seen *in vitro* have all been found by careful and prolonged search of stained thin films made daily from rats and monkeys infected with *T. rhodesiense* by cyclically infected *G. morsitans*. Plate XV, figs. 1–8, are photomicrographs of the forms found in stained thin films, and show the sequence of events. The difference in the character of the nuclei in the fusion forms should be noted.

With regard to our *in vitro* findings in *T. rhodesiense*, Hoare (1936) has given a very similar description of the behaviour of *T. congolense* in fresh-blood preparations—though he refers to the fusion of two trypanosomes as 'auto-agglutination or agglomeration'—and discusses its bearing on the fusion forms seen so frequently in *T. simiae*. His final remark, however, is: 'Finally, lest there might be any temptation to interpret the phenomenon described above as a sexual process, it should be emphasized that in no case have any nuclear changes been observed in the agglutinating flagellates and, moreover, the union may take place indiscriminately between two non-dividing forms . . . between these and dividing forms . . . between dividing forms, and between more than two individuals.'

The fact that Hoare observed no agglutinating flagellates with fused nuclei was probably due to the comparative rarity of this stage, which we think persists only for a

matter of seconds, and to the fact that he was examining single slides sent to him from the field. We obtained a strain of *T. simiae* from a naturally infected horse at Old Shinyanga, and passaged it for seven months by *G. morsitans* through monkeys and sheep, and this gave us ample material for the study of daily thin films made from any one host. Plate XVI, figs. 9-17, are photomicrographs of the forms of *T. simiae* found in stained films from various monkeys, and clearly show the sequence of events.

The above description refers to fusion in the 'head-to-tail' position; but 'head-to-head' fusion has also been seen to occur *in vitro*, particularly in the case of the long forms. The process in *T. rhodesiense* is illustrated in Plate XVII, figs. 18-24.

In *T. simiae* we have observed an identical process. Sometimes, however, one of a pair of trypanosomes appeared to be a 'ghost,' staining less deeply than the other and exhibiting no sign of a nucleus. Plate XVIII, figs. 25-27, show these forms. On photographing them, however, with a green filter and a panchromatic film, the suggestion of a nucleus was sometimes to be seen in the final print (fig. 27).

On heavily restaining the slides we found that trypanosomes in which the nucleus had hitherto been invisible now showed nuclear-staining material. The fact that the nucleus of the 'ghost' was only stained with such difficulty is evidence of its being in a different state from the other of the pair, and suggests that these 'head-to-head' forms were not in a state of division or agglomeration.

In the past, 'head-to-head' fusion in *T. simiae* has been taken as an aberrant type of division (Bruce *et al.*, 1914) or as auto-agglutination or agglomeration (Hoare, 1936); but these views are not tenable. Because of the nuclear changes which we have found in both *T. rhodesiense* and *T. simiae*, we are unable to reach any conclusion other than that the process we have observed is syngamy, which can apparently occur in warm mammalian blood *in vivo*, in cooled living preparations, and in the tsetse-fly, as will be shown in the next section.

In the fusion forms of both *T. rhodesiense* and *T. simiae* there will be seen examples either of a single kinetoplast or of two kinetoplasts joined by a line (see Plate XVI, figs. 15-17, and Plate XVII, fig. 24). The exact significance of this is not clear.

XIII. THE SIGNIFICANCE OF SYNGAMY

The authors felt strongly that this attempted synthesis could never satisfy unless the phenomenon of syngamy in *T. rhodesiense* were placed in its morphological, physiological and genetic context; and so they set to work to discover, if possible, what causes syngamy to take place, what is its physiological function in a trypanosome, and what are its genetic and morphological implications. In this description of the work, we have deemed it best to describe our experiments in more or less chronological order, and to take the reader along the same road of reasoning we ourselves travelled.

The first question that we sought to answer was 'Which of the three forms enters into syngamy with which, and what effect does sign of charge produce?' We found that syngamy could be induced in blood trypanosomes by almost any marked and sudden change of environment. Trypanosomes from a rat would enter into syngamy if placed in Ringer-glucose solution, citrated saline, normal saline, phosphate buffers and glucose saline, provided that there was insufficient glucose present to cause adherence between the trypanosomes and the red blood-corpuscles. Syngamy could also be observed in infected blood withdrawn from the crop of a tsetse-fly. In all these media we found that each form

—the long, the intermediate and the short—could fuse with itself or with either of the other two forms. In other words, if *T. rhodesiense* has sex, as Vanderplank (1944) suggests, then sex and morphology are not obviously related. Furthermore, his suggested mechanism of fertilization by gametes extruded from one trypanosome and entering another cannot be valid, unless we are prepared to postulate two entirely different methods of syngamy in the same organism, which is more than unlikely.

To determine whether any relationship existed between the sign of the charge on a trypanosome and its ability to enter into syngamy, we separated the negative variant of *T. rhodesiense* by suspending infected rats' blood in Broom, Brown and Hoare's glucose-saline solution. The positively charged trypanosomes adhering to the red blood-corpuscles were then precipitated by centrifuging, and the supernatant liquid, containing the negatively charged trypanosomes, was pipetted off and examined in a 0.1 mm.-deep cell. Repeated attempts to demonstrate syngamy in this variant failed, and we therefore concluded that the positive variant is essential to syngamy.

We found that shaking up the sediment of positively charged trypanosomes and adherent red blood-corpuscles with an excess of citrated saline had no appreciable effect in freeing the trypanosomes. By the addition of Ringer-glucose solution to the sediment, however, the trypanosomes were readily freed, and we found that in 15–20 minutes syngamy had started.

Unfortunately, it was impossible to be certain that the addition of Ringer-glucose solution, in order to free the trypanosomes from the adherent red blood-corpuscles, had not changed the charge on some of the former. Four tubes of positively charged trypanosomes resuspended in Ringer-glucose solution were therefore set up. At intervals of 5, 20, 35 and 50 minutes a tube was selected, and glucose-saline solution (to which had been added a small amount of sheep serum, which previous experience had shown to encourage adherence) was added, and the specimen was examined. The results were:

After	Percentage of adherent trypanosomes
5 minutes	70
20 "	61
35 "	76
50 "	88

This experiment shows that the positive variant, separated by the glucose-saline solution and resuspended in Ringer-glucose solution, does not remain purely positively charged, and the syngamy that we observed may have been due to the presence of a mixture of positively and negatively charged trypanosomes. Up to date we have been unable to produce a perfectly pure positive variant free from adherent red blood-corpuscles, and the question of whether syngamy can occur in such a variant remains unanswered for the present. In any event, syngamy between trypanosomes carrying opposite charges cannot be regarded as a sexual process, since the sign of the charge on a trypanosome can be reversed by chemical means.

The next stage in our investigation consisted in an attempt to tie up the process of syngamy with the production, and the fluctuation in the proportion, of the blood forms.

Muriel Robertson (1913*b*), working with *T. gambiense*, stated that the short forms did not divide, but elongated into long trypanosomes which were the dividing forms. She did not, however, explain how the short forms were replenished in the blood. We have found a very few short forms of *T. rhodesiense* in the blood of one monkey which might suggest binary fission (Plate XIX, figs. 31 and 32). It will be noticed, however,

that they depart fundamentally from the classic description of this process; and on this account, and also because we have seen similar forms produced *in vitro* by the fusion of two trypanosomes, we consider them as probably instances of 'head-to-head' syngamy.

Four hundred dividing forms of *T. rhodesiense* from rat and 186 from monkey were measured, and the lengths gave the distribution curves shown in fig. 7. The means were $31.385\mu \pm 0.144\mu$ for rat, and $30.694\mu \pm 0.208\mu$ for monkey, which were rather longer than the mean for the long form in rat, as determined above. Neither curve showed any significant departure from the normal Gaussian type, g_1 being -0.126 ± 0.122 for rat and 0.250 ± 0.178 for monkey, while g_2 was 0.162 ± 0.243 for rat and 0.0085 ± 0.355

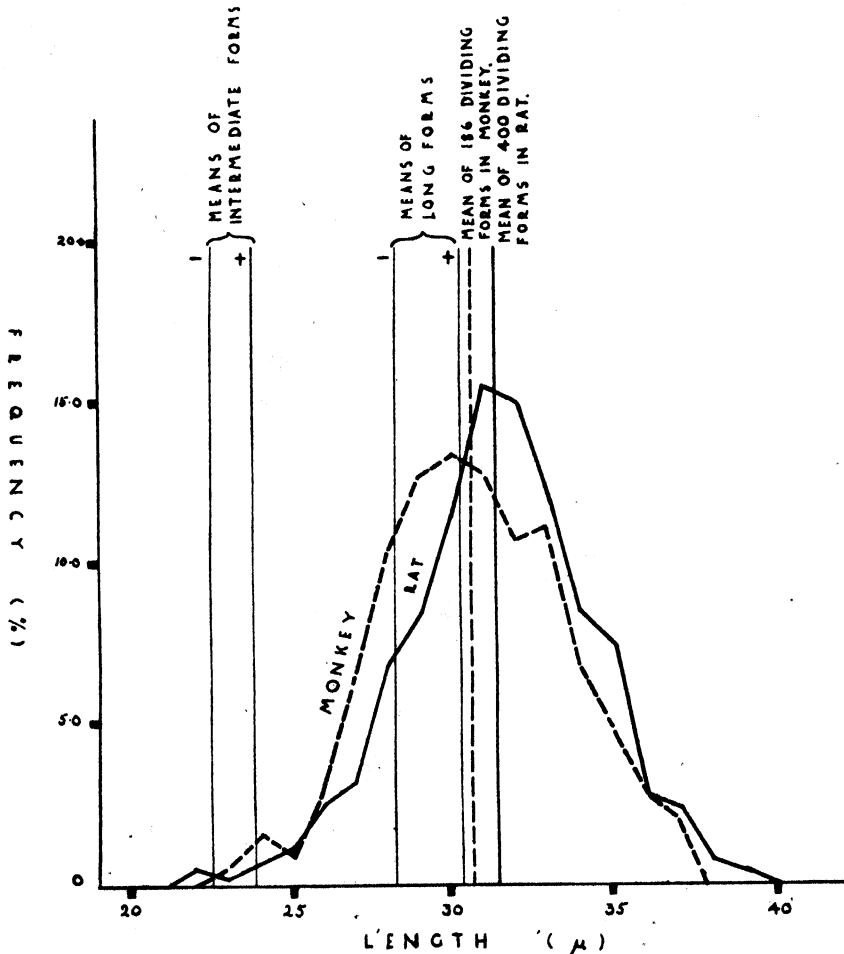


FIG. 7. The length distribution of dividing forms of *T. rhodesiense* in monkey and rat.

for monkey. It was therefore obvious that these statistical populations did not include any appreciable numbers of intermediate or short forms, and we therefore concluded that they were certainly composed almost entirely, and possibly completely, of the long form, and that this is the only one which divides in any appreciable numbers in the blood

—a conclusion strongly supported by a careful study of the morphology of dividing trypanosomes.

The length distribution of the trypanosomes present in the blood of rat 6503 (*supra*) on the second, fourth and sixth days of the infection is shown in fig. 8. The proportion of short forms present was respectively 1.9 per cent., 18.9 per cent. and 50.8 per cent. ; and, as the percentage of short forms increased, the length distribution of the remaining trypanosomes shifted markedly into the region normally occupied by the intermediate forms. As the production of short trypanosomes was accompanied by a corresponding increase in the intermediate forms, and since neither of these types divide in any numbers, if at all, we came to the conclusion that they were being produced from the long forms. A re-examination of the slides showed that during this period long trypanosomes in syngamy were not infrequent.

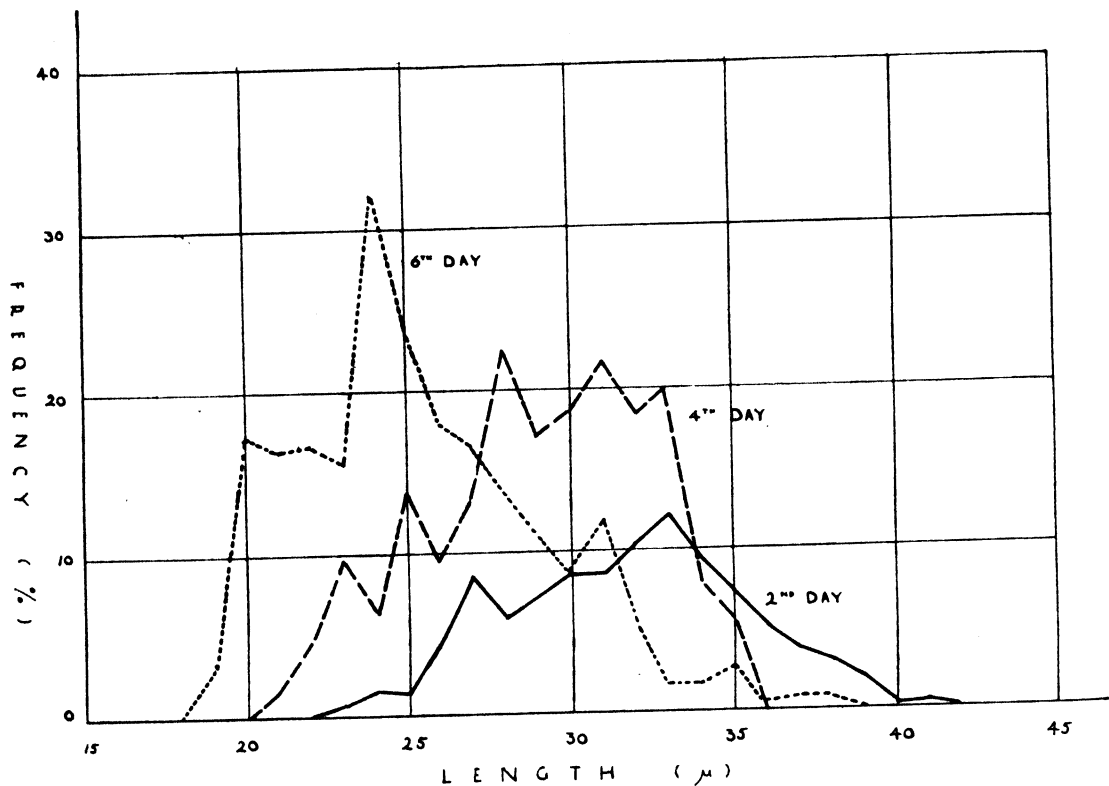


FIG. 8. The length distribution of the trypanosomes in rat 6503 on the second, fourth and sixth days.

Two *G. morsitans* were fed on rat 7699, and the proportions of the various types of trypanosomes present in the blood of the rat, and in the blood extracted from the crops of the flies after 30 minutes and three hours, were determined. The rat's blood contained 77 per cent. of long forms, 9 per cent. of short forms, and 14 per cent. of intermediate forms. After half an hour in the crop of the fly there were only 30 per cent. of long forms present, the remainder being short and intermediate forms in approximately equal proportions. It was unfortunately impossible to obtain accurate figures for these latter two

forms, because in this transition stage the morphological differences between them tended to be indistinct, and so there were many border-line cases.

Comparing the blood taken from the crop of the fly after half an hour with that of the control, it was found that the number of trypanosomes per hundred red blood-corpuscles had remained constant, showing that the increase in the proportion of the short and intermediate forms could not be attributed to a heavy mortality amongst the long forms. There were a few dividing long forms present on both slides, but no dividing short or intermediate forms could be found, and there was marked evidence of syngamy in the blood from the crop.

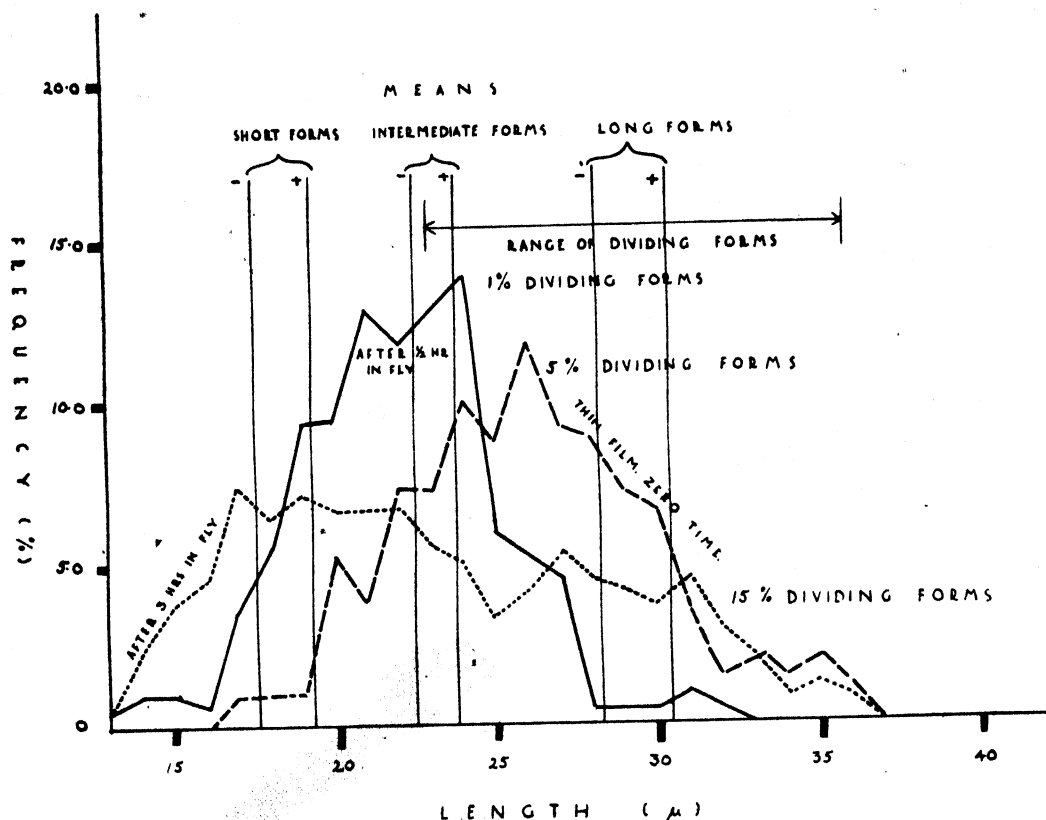


FIG. 9. The length distribution of the trypanosomes in rat 7699 and in the crops of tsetse-flies fed on it.

The blood withdrawn from the crop of the second fly after three hours was smeared, fixed and stained, and there was found a large number of long dividing forms (15 per cent.) ranging from 23μ to 36μ , but no short or intermediate forms in division. In spite of much degeneration of the long trypanosomes, and comparatively little of the others, the slide showed a far higher proportion of long forms than did the blood taken after half an hour in the fly. The length distribution of the trypanosomes is shown in fig. 9, which brings out the shift in length during the experiment.

When rat 7693 was examined there were only 5 per cent. of long forms in 198 trypanosomes measured. A drop of this rat's blood was mixed with normal citrated saline on a slide, and the slide was placed in a saturated aqueous atmosphere. After five minutes

the mixture was smeared, fixed and stained, when it was found that many of the trypanosomes were in syngamy and that the proportion of long forms had risen to 28 per cent.

Two *G. morsitans* were fed on infected rat 7718 at a time when 17 per cent. of the trypanosomes were short forms. After half an hour blood was withdrawn from the crop of one fly, and it was found that the proportion of short forms was now only 2 per cent., that there were short and/or intermediate forms in syngamy and a number of long forms in syngamy, and that there were a number of dividing long forms but no dividing short forms.

In the blood withdrawn after three hours from the crop of the other fly there were 21.5 per cent. of short forms, the blood was full of dividing long forms, but no dividing short or intermediate ones, and there were many long forms in syngamy in the 'head-to-head' position and some short and intermediate forms in syngamy in the 'head-to-tail' position.

Similar phenomena were readily produced merely by cooling a drop of blood in a saturated aqueous atmosphere for 10-20 minutes; on smearing, fixing and staining, trypanosomes in syngamy were found.

The long trypanosome can multiply by binary fission, but apparently the short and intermediate ones cannot do so readily, if at all; and the above experiments lead to the conclusion that these latter two forms are produced by syngamy between the long forms. Syngamy between the short and intermediate trypanosomes produces long forms, which may either multiply by binary fission or re-enter into syngamy.

One of us (H. F.) had measured a large number of metacyclic trypanosomes of *T. rhodesiense*, obtained by inducing infected *G. morsitans* to probe on to albumen-covered slides. The lengths of these metacyclic trypanosomes were not distributed normally. It was seen, however, that the metacyclics were of two types, those with, and those without, a flagellum, and, when the lengths of these types were analysed separately by Mr. F. L. Gee, it was found that each gave a Gaussian distribution, and that the two means were significantly different. Further, though the proportion of the two types might vary from fly to fly, they were equal when averaged over a number of flies. This work will be published in fuller detail elsewhere.

We thus have two metacyclic forms which tend to occur in equal numbers, and these give rise to three blood forms, each with two electrical variants. In the first stages of the disease, the blood infection as a rule consists predominantly of the long, positively charged trypanosome, the mean length of which, as determined by Gee, is 30.380μ . Later the other forms appear, with means of 17.555μ , 19.320μ , 22.65μ , 23.897μ and 28.24μ , and throughout the disease the proportions of these six types fluctuate widely in the blood.

The length and shape of *T. rhodesiense*, the type of reproduction by binary fission, and the pseudo-alternation of generations in the fly and the mammalian host, are all associated together, and the maintenance of this association is necessary for the reproductive cycle. Where sex is fully developed, a similar association is maintained by lack of pairing between the sex chromosomes, and it is suggested that in the case of *T. rhodesiense* also two chromosomes remain unpaired.

If the unpaired chromosomes are A and A', a combination of these would account for the three blood forms, AA, A'A' and AA'. As the long thin form of the trypanosome is the predominant one in any infection, as it is the only one which reproduces readily by binary fission—perhaps the only one which reproduces in this way at all—and as the

other two types are produced from it by syngamy, it must contain both types of chromosome, being heterogeneous, and have the structure AA'. The vigour which this form displays is consistent with heterosis. The short and intermediate forms can only then be AA and A'A'.

Oehler (1913) reported a method for inoculating animals with single trypanosomes, but he had only 10 successes in 31 inoculations, using four different strains (species unstated). Later (Oehler, 1914) he was successful on two out of 40 occasions, using a strain of *T. brucei*. In every infection from the inoculation of a single flagellate, the trypanosomes developed typical polymorphism (Prowazek, 1913, *T. rhodesiense*; Oehler, 1914, *T. brucei*); and this could only have been possible if the trypanosome inoculated had been the heterogeneous form, AA'. The numerous failures to infect would also be explicable on the grounds that the single trypanosomes used on these occasions were homogeneous, AA or A'A', which do not divide and which were therefore unable to multiply, and, being single individuals, could not enter into syngamy with another trypanosome to produce the heterogeneous dividing form AA'.

In the arm-reactions of men, and in the early blood infection of mammals, the first trypanosomes to be seen are, as a rule, the long heterogeneous form, AA'. Their formation from two different types of metacyclic trypanosomes can most easily be explained if we assume that the latter consist of the homogeneous types, AA and A'A', producing by syngamy the heterogeneous dividing form, AA', syngamy being stimulated, as we have shown, by any abrupt change of environment.

Such evidence as we have, though not conclusive, suggests that syngamy probably takes place between trypanosomes of opposite charge, and if the metacyclic trypanosomes enter into syngamy when inoculated by the fly into a mammal, as seems to be the case, then one would expect them to be both positively and negatively charged.

When Broom and Brown (1937) were examining the developmental forms of *T. brucei* in the tsetse-fly, they came to the conclusion that the saliva prevented adhesion between the trypanosomes and the red blood-corpuscles in their glucose-saline solution. To overcome this difficulty, and also because they found that the metacyclic trypanosomes did not survive in glucose-saline, they washed them in glucose-Ringer solution, resuspending them in the same solution with the addition of a drop of mouse blood, centrifuged at 7,000 revolutions per minute for 20 minutes, and examined the deposit (Broom and Brown, 1939). With this technique they found that all the metacyclic trypanosomes were positively charged and adherent to the red blood-corpuscles; but they also remarked that on many occasions they failed to find any trypanosomes in the deposit, and they do not appear to have examined the supernatant liquid, which was pipetted off, to see if it contained any free-swimming forms.

We induced infected flies to probe into drops of Broom and Brown's glucose-Ringer solution (10 per cent. Ringer in 4 per cent. glucose), to which clean rat's blood had been added, and we found that of 108 metacyclic *T. rhodesiense* counted 89 were free-swimming and only 19 adherent. This result might have been due either to the action of the saliva preventing adherence (see above) or to the Ringer component of the solution altering the charge on the trypanosomes, as we have shown may occur.

We therefore induced an infected fly to probe into a drop of defibrinated sheep blood, which was then smeared, fixed and stained. The form of the red blood-corpuscles showed that they were still charged; and, of 100 trypanosomes examined by this thin-film method,

90 were positively charged and 10 negatively charged. We consider that this technique gives a truer picture of the electrical condition of the metacyclic trypanosomes on being inoculated into an animal.

The argument that all the trypanosomes of *T. rhodesiense* probably have *two* unpaired chromosomes is in contradiction to Vanderplank (1944), who stated that there were two chromosome patterns, the short forms having two pairs of chromosomes and one unpaired one ($N = 5$), and the long thin trypanosomes having two pairs of chromosomes and two unpaired ones ($N = 6$). If this were the case, then in the two types of metacyclic trypanosomes one form should have five and the other six chromosomes; but, if that were so, these two types would never be able to give rise to three types, and three only, in the blood.

We therefore attempted to repeat Vanderplank's work, but could not achieve any success by his methods, and so sought to evolve another technique.

There were two main difficulties. In the first place, only a small fraction of trypanosomes in a sample is in a nuclear state in which chromosomes are visible. Secondly, the nucleus of *T. rhodesiense* is small, and this raises grave optical difficulties connected with the limits of resolution.

It was argued that the proportion of nuclei in a suitable state might be increased by subjecting the organism to conditions known to encourage syngamy, and that optical resolution might be aided if the nucleus could be expanded. We therefore suspended trypanosomes in the harsh environment of normal citrated saline diluted with its own volume of distilled water, and found that they would remain motile in this for at least 20 minutes at room-temperature (25° C.). They also readily entered into syngamy, while the hypotonic condition of the solution caused liquid to enter the flagellate, which increased markedly in breadth.

Drops of blood from infected rats were placed on slides, mixed with a drop of this diluted citrated saline solution, and the slides were then placed in a saturated aqueous atmosphere for five or ten minutes, smeared, fixed in alcohol, stained lightly with Giemsa stain, quickly rinsed and dried.

It was found that this technique had the desired effect, and that chromosomes were visible in some of the trypanosomes. Resolution was, however, only possible when the chromosomes happened to be more widely separated than occurred on the average, and these cases were rare, but were drawn when found.

Photography was even more difficult, because, owing to the high magnification needed, good definition was only possible when all the chromosomes lay in one horizontal plane, so that the number of trypanosomes which could be successfully photographed was extremely small. Plate XIX, figs. 33 and 34, show some of the results.

It was found that in all forms the chromosome number was six. No trypanosomes with five were found, though in some cases one or more chromosomes stained more faintly than the rest, so that they could easily have been overlooked. Sometimes, too, one or more overlay another, or lay so close, as to give an impression of there being five, four, or even only three.

Syngamy in *T. rhodesiense* is not a typical copulation in which two gametes unite completely. In the first place, each one of the fusing pair retains its own identity to some extent throughout the process, and secondly, since one form of the normal trypanosome with six chromosomes exhibits heterosis, $N = 6$ must represent the diploid phase of the organism, and hence the fusing pair cannot be considered as gametes.

The occurrence of genetic segregation for size and form implies meiotic division, indications of which are to be seen in the nuclear structure revealed in some of the photomicrographs reproduced.

We consider that syngamy, which in our experience is invariably associated with change of environment, points to its being part of an adaptive mechanism in which two flagellates associate to exchange a group of three chromosomes.

Trypanosomes in syngamy, observed *in vitro*, have frequently been seen to extrude granules (Plate XIX, figs. 35-37). These are usually liberated just before the pair finally separate, though in some cases the extrusion takes place after separation, but never, so far as our observations go, in any of the earlier phases of syngamy. In spite of repeated observations, we have never seen one of these granules enter another trypanosome, and cannot therefore agree with Vanderplank (1944) that they are gametes. Leaving behind, as they do, a diploid nucleus, they cannot be normal reduction bodies, and their nature, which is obscure, requires further investigation.

It should not be forgotten that syngamy in *T. rhodesiense*, while it may enable a strain to adapt itself to a new environment, and so ultimately accelerate multiplication by binary fission, may for a time actually reduce the rate of increase considerably by converting long forms into the two homogeneous types, which, as we have seen, divide extremely rarely, if at all.

We should therefore not expect syngamy to occur except when the stress of the environment makes it imperative for the maintenance of the strain. For instance, syringe-passaged strains, in which subinoculations are made from animals as soon as their blood proves microscopically positive, are subjected to none of the environmental stresses which affect a cyclically transmitted strain. Syngamy in these circumstances would have no survival value; on the contrary, it would merely retard multiplication, and trypanosomes which entered into syngamy, once the strain had become thoroughly adapted, would not be on equal terms with less 'fertile' ones, which in nature would, of course, tend to die out. Therefore, while in nature the capacity to produce short and intermediate forms by syngamy of the long forms is essential to maintaining the strain by ensuring its plasticity, a syringe-passaged strain, in which the subinoculations are made early in the infection, should be able to exist perfectly well as the pure, long, heterogeneous form, AA', maintaining itself solely by vegetative division in an ideal artificial environment where plasticity is not necessary.

The tendency would therefore be to breed out the more adaptable forms, i.e., those most capable of syngamy, selection encouraging the multiplication of those with the highest rate of binary fission and tending towards a fixation of properties, e.g., virulence, and also to a loss of the capacity for changing the sign of the electrical charge, which, as we have seen, may be closely connected with syngamy and certainly seems to be a property necessary to successful cyclical transmission.

The end-point of this process is to be seen in certain of the 'fixed' strains maintained for years in European laboratories by syringe passage, which, however interesting they may be, are most limited in their usefulness for studying the naturally occurring organism.

Syngamy in trypanosomes is obviously a matter of some practical importance, in that it opens up the possibility of breeding strains artificially for different purposes.

The authors are aware that in this paper they have developed an elaborate thesis. They have done so in the hope that this attempted integration of the subject by a new

approach to it, this tentative co-ordination of random facts which have been unexplained and unrelated for so long, may stimulate others either to confirm and amplify their views or to carry out investigations designed to provide us with a sounder synthesis.

APPENDIX

STATISTICAL ANALYSIS

by

F. L. GEE

1. The distribution of the lengths for the sample of *T. rhodesiense* described in the main paper are given in Table III, together with graduations of them on various hypotheses and the values for P resulting from tests of these hypotheses.

2. The method used for dissecting a distribution into two Gaussian components was as follows. Let the number of observations, their mean and their moments about their mean be $N, M, \mu_2, \mu_3, \mu_4, \mu_5$, and let the number of individuals, the means and standard deviations of the components be n_1, m_1, σ_1 , and n_2, m_2, σ_2 . Then, equating moments and writing $d_1 = M - m_1, d_2 = M - m_2$, and, where convenient, $x = d_1 d_2, y = d_1 + d_2$, we obtain, after suitable elimination:

$$n_1 = \frac{Nd_2}{d_2 - d_1} \quad \dots \dots \dots (i)$$

$$n_2 = \frac{Nd_1}{d_1 - d_2} \quad \dots \dots \dots (ii)$$

$$\sigma_1^2 = \mu_2 + x - \frac{d_1}{3} \left(\frac{\mu_3}{x} + y \right) \quad \dots \dots \dots (iii)$$

$$\sigma_2^2 = \mu_2 + x - \frac{d_2}{3} \left(\frac{\mu_3}{x} + y \right) \quad \dots \dots \dots (iv)$$

$$y = \frac{-\mu_3 \mp \sqrt{3x^3 - \frac{3}{2} \left(3\mu_2^2 - \mu_4 \right) x + \frac{3}{2} \mu_3^2}}{x} \quad \dots \dots \dots (v)$$

$$\begin{aligned} & 3\mu_3 \left\{ x^3 + \left(5\mu_2 - \frac{1}{2} \frac{\mu_3}{\mu_2} \right) x^2 + \frac{3}{2} \left(3\mu_2^2 - \mu_4 \right) x - \mu_3^2 \right\} \\ & = \pm \left\{ x^3 - \frac{3}{2} \left(3\mu_2^2 - \mu_4 \right) x + 2\mu_3^2 \right\} \sqrt{3x^3 - \frac{3}{2} \left(3\mu_2^2 - \mu_4 \right) x + \frac{3}{2} \mu_3^2} \quad \dots \dots (vi) \end{aligned}$$

Equation (vi) is solved by trial. Only one of the solutions of (v) and (vi) yields values for d, n and σ which satisfy the moment equations relevantly. The details of the components of thin + and thin - are given in Table IV.

3. The distribution of lengths in a homogeneous population of living creatures is usually Gaussian or nearly so, and where a distribution of this kind departs from the Gaussian it can usually be represented by a Pearson curve.

TABLE III

Mean σ β_1 β_2	Stout — 17.555 2.042 0.00540 3.096		Thin + 28.960 4.465 0.000622 2.554			Thin — 26.372 4.535 0.192 2.684			Stout + 19.320 2.011 0.0415 3.305		
Length in μ	Observ- ed	Gaussian	Observ- ed	Pearson type II	Double Gaussian	Observ- ed	Pearson type I	Double Gaussian	Observ- ed	Gaussian	Pearson type IV
8		0.0									
9		0.1									
10		0.3								0.0	0.0
11		1.3								0.1	0.1
12	8	5.4						0.0		0.5	0.4
13	15	18.0			0.0			0.1		2.6	1.9
14	45	49.3		0.0	0.1			0.2	15	10.9	8.4
15	105	93.0		0.2	0.4			0.6	23	34.7	29.9
16	144	154.9		1.7	1.6			1.6	71	87.3	83.8
17	212	199.7	7	7.0	4.8	4	1.3	2.7	175	173.3	178.7
18	203	201.4	10	18.8	13.1	14	11.8	7.4	312	269.4	285.5
19	157	161.6	34	39.5	31.4	11	24.5	16.4	349	328.9	341.9
20	92	102.4	62	69.9	64.0	40	38.8	31.7	293	314.8	311.4
21	54	51.1	129	110.0	113.7	39	50.7	52.1	229	236.3	221.7
22	22	20.1	182	158.3	175.2	67	60.0	70.7	111	139.0	127.6
23	8	6.4	224	212.2	237.6	84	65.9	77.8	67	64.4	61.8
24	2	1.6	278	267.5	288.3	77	69.0	75.1	32	23.4	26.0
25		0.3	324	320.2	323.4	65	69.3	66.6	8	6.7	9.8
26		0.1	323	366.4	347.7	65	67.4	61.0	6	1.5	3.5
27		0.0	382	402.2	369.9	49	63.7	54.9		0.2	1.2
28			395	424.9	394.9	57	58.6	52.9		0.0	0.3
29			453	432.1	417.8	59	52.4	50.8			0.1
30			399	423.7	429.0	45	45.8	48.1			0.0
31			447	399.9	419.8	34	38.9	42.6			
32			385	363.1	387.1	34	32.0	34.9			
33			335	316.1	334.4	35	25.7	27.8			
34			267	262.9	270.3	17	19.9	21.8			
35			195	207.7	204.3	17	14.9	14.8			
36			130	154.2	144.2	10	10.8	10.1			
37			94	106.4	95.0	7	7.3	6.6			
38			65	67.0	58.6	3	4.5	3.9			
39			40	37.6	33.8	3	2.6	2.4			
40			15	17.6	18.2	0	1.4	1.2			
41			15	6.4	9.1	1	0.6	0.6			
42			5	1.4	4.3	1	0.2	0.3			
43				0.1	1.9		0.0	0.2			
44				0.0	0.7			0.1			
45					0.3			0.0			
46					0.1						
47					0.0						
Total	1,067		5,195			838			1,694		
χ^2		5.44		42.25	18.43		28.73	18.08		29.82	16.37 [10.93]
n		10		23	24		20	20		11	12 [11]
k		3		4	6		5	6		3	5
P		0.61		0.0016	0.43		0.018	0.20		0.00023	0.022 [0.125]

(a) Tail grouping for the χ^2 test is indicated by long brackets. The figures in square brackets for stout + result from including the 23 observations for length 15μ in the lower tail.

(b) The type II curve for thin + starts at 13.857μ and finishes at 44.064μ . The type I curve for thin — starts at 17.09μ and finishes at 45.01μ ; its mode is at 24.64μ . The mode of the type IV curve is at 19.142μ .

TABLE IV

	Thin +		Thin —	
	Intermediate	Long	Intermediate	Long
n	1,137.4	4,057.6	280	558
m	23.897	30.380	22.65	28.24
σ	2.4658	3.8239	2.035	4.300

Comments on the Graduations in Table III

Stout —. Closely Gaussian.

Stout +. The very poor fit of the Gaussian curve is due to a skewness of borderline significance (Sk , 0.0883; σsk , 0.0306), combined with a small leptokurtosis (γ_2 , 0.305; $\sigma \gamma_2$, 0.238). The type IV gives a fair fit, the actual value for P depending to an abnormal extent on the grouping for the lower tail.

Thin —. This is very significantly skew and fairly platykurtic. Formally, there is no double-Gaussian graduation, but an alteration in μ_s of the order of 0.1 of its standard error would provide a solution. The solution actually given is that which arises from the value of x which most nearly solves equation (vi). This gives quite a good fit and represents the modes well. The type I curve gives a single mode in the wrong place, and its general fit is rather poor.

Thin +. This is insignificantly skew but very significantly platykurtic. The double Gaussian not only gives an excellent fit, but also represents well the mode at about 30μ and the 'submerged mode' at about 25μ . Type II ignores these modes and, as a whole, gives a very poor fit.

4. *Summary of Conclusions.* There appears to be weighty evidence that thin + consists of two components. The same applies with rather less weight to thin —. Although there may be a small amount of heterogeneity in stout +, there is no evidence that it is not in the main a distribution of a single type. There is no evidence that stout — is not a single type. Thus, it seems very probable that the whole sample divides into six main forms, viz., stout —, stout +, thin — intermediate, thin — long, thin + intermediate, and thin + long.

SUMMARY

In Part I the blood forms of trypanosomes are treated as electrically charged bodies moving amongst the negatively charged red blood-corpuscles. It is pointed out that, if the charges involved produce appreciable electrostatic fields at the distances we are here dealing with, certain results will follow; and on this basis the following predictions are made: (a) trypanosomes will not be distributed at random on a thin blood film; (b) the mean length of a negatively charged variant of a trypanosome will be less than that of the corresponding positively charged one.

From a consideration of the various changes which trypanosomes undergo, it is predicted that they will be found to enter into syngamy.

In Part II it is shown experimentally that all these predictions are accurate.

By measuring the mutual deformation produced when two red blood-corpuscles approach one another, it was demonstrated that they exert a mutually repulsive force

following the inverse square law—a property which is not in accord with the usually accepted views on diffusion equilibria, and which seems to be associated with the biological activity of the cell, since on 'killing' the latter the repulsion ceases.

Trypanosomes on a thin blood film are not distributed at random, but lie in relation to the red blood-corpuscles according to the charge carried; and a method, based on this fact, is described for determining the sign of the charge on a trypanosome by examination of a thin blood film.

Using this method, and dividing the trypanosomes (*T. rhodesiense*) into 'stout' forms (i.e., short fat forms with no free flagellum, or only a short one) and 'thin' forms (i.e., long thin forms with a marked free flagellum), 8,794 trypanosomes were measured in 15 consecutive daily thin films from a rat infected by the bite of a tsetse-fly.

Statistical analysis of these measurements showed that there were three main forms of *T. rhodesiense*, each existing as a positively and a negatively charged variant, giving six types in all. In each case the mean length of the negatively charged variant was, as predicted on theoretical grounds, significantly shorter than that of the corresponding positively charged variant.

The long and intermediate forms can be differentiated morphologically by the shapes of their posterior ends. When trypanosomes, separated by this criterion, were measured, each form gave a Gaussian length-distribution curve, and the mean of each corresponded closely with the values obtained by statistical analysis of the composite sample mentioned above.

Posterior-nuclear forms of *T. rhodesiense* have been found in the blood of man, and photographs of them are reproduced.

Syngamy has been found to take place in *T. rhodesiense* and *T. simiae*. The process is described, and photographs are reproduced illustrating the sequence of events and showing forms with the nuclei fused.

It was found that the long thin form, with means at 28.24μ and 30.380μ , was the only one which reproduced to any extent by binary fission, the short and intermediate forms being produced by syngamy.

It is suggested that syngamy takes place only between trypanosomes carrying electrical charges of different sign, but this point has not been finally established.

An infected fly was induced to probe into a drop of defibrinated blood, and the charge of the metacyclic trypanosomes was determined by the thin-film method. It was found that only 90 per cent. were positively charged, which is contrary to Broom and Brown, who found by a different method that they were all positively charged.

As the length and shape of *T. rhodesiense*, the type of reproduction by binary fission, and the pseudo-alternation of generations in the fly and the mammalian host are all associated together, and since the maintenance of this association is essential to the reproductive cycle, and as a similar association is, where sex is developed, maintained by a lack of pairing between sex chromosomes, it is postulated that in *T. rhodesiense* also two chromosomes remain unpaired.

It has been shown that the metacyclic trypanosomes of *T. rhodesiense* occur as two distinct types, each with a Gaussian length distribution and with significantly different mean lengths. These two types produce three blood types. It is suggested that the genetic structure of the metacyclic trypanosomes may be denoted as AA and A'A', while the blood forms are AA, A'A' and AA'.

As the long form is the predominant one in any infection, as it is the only one which reproduces readily by binary fission, and as the short and intermediate forms are produced from it by a process of syngamy, it must be heterogeneous, AA'.

The small percentage of success achieved by previous workers in attempts to infect rats with single trypanosomes from recently isolated natural strains is explained by their failure to inoculate the heterogeneous form, AA', a single trypanosome of either of the other two forms being obviously incapable of infecting.

Vanderplank's work on the chromosome structure of *T. rhodesiense* was repeated, and it was found that, contrary to his results, all forms had six chromosomes, as one would expect on theoretical grounds.

A technique is described for enlarging the nucleus of a trypanosome to render the chromosomes resolvable.

Bodies being extruded from the nuclei of trypanosomes in syngamy, and from other trypanosomes, are illustrated. Their exact nature requires further elucidation.

The implications of syngamy are discussed. The development of monomorphic non-transmissible strains in European laboratories by prolonged syringe passages is explained, and reasons are given why the results obtained by work on such strains are not necessarily applicable to naturally transmitted strains.

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The forms illustrated in Plates XV-XIX were photographed from thin blood films which had been fixed with alcohol and stained with Giemsa stain, with the exception of figs. 28-30, which were from Giemsa-stained thick films. The magnification is approximately $\times 2,000$.

SYNGAMY IN *Trypanosoma rhodesiense*—'HEAD-TO-TAIL' POSITION



FIG. 1



FIG. 2

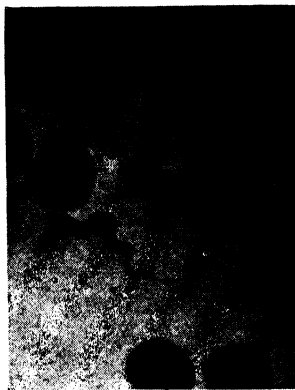


FIG. 3



FIG. 4

FIGS. 1-4. Two trypanosomes approaching and lying parallel to each other. (Note the nucleus present as a bar in fig. 4.)



FIG. 5



FIG. 6

Two trypanosomes with a single nucleus.



FIG. 7

Trypanosomes in syngamy. It is impossible to say whether their nuclei are about to fuse or are in the process of separation.



FIG. 8

The two trypanosomes separating, sliding apart anteriorly from each other.

(Note the nuclear structure in many of the trypanosomes illustrated.)

SYNGAMY IN *Trypanosoma simiae*—'HEAD-TO-TAIL' POSITION

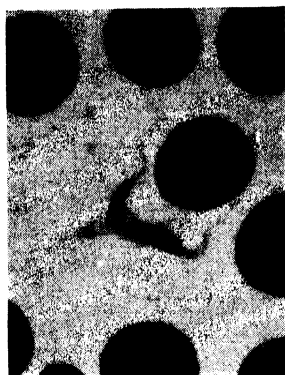


FIG. 9

Two trypanosomes intertwined.



FIG. 10

FIGS. 10-11. The trypanosomes fused, with nuclei opposed.

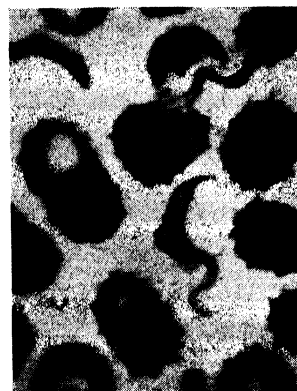


FIG. 11



FIG. 12



FIG. 13

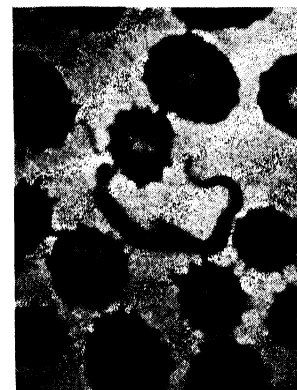


FIG. 14

FIGS. 12-14. Two trypanosomes fused with a single nucleus. (In both figs. 13 and 14 there is present a third normal trypanosome touching the forms in syngamy.)

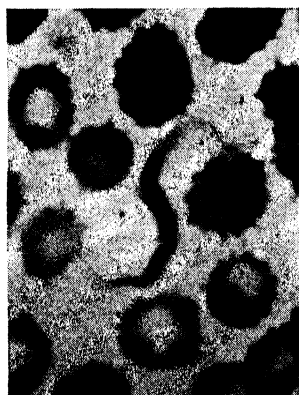


FIG. 15

Two adherent forms with a single kinetoplast.



FIG. 16



FIG. 17

FIGS. 16-17. Note the line connecting the two kinetoplasts. In fig. 17 there is also present a form in normal binary fission.

SYNGAMY IN *Trypanosoma rhodesiense*—'HEAD-TO-HEAD' POSITION



FIG. 18

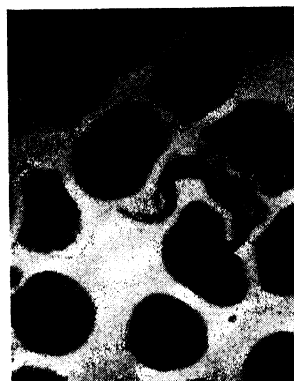


FIG. 19

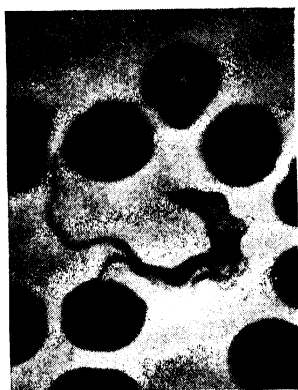


FIG. 20

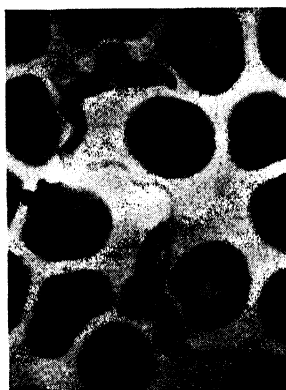


FIG. 21

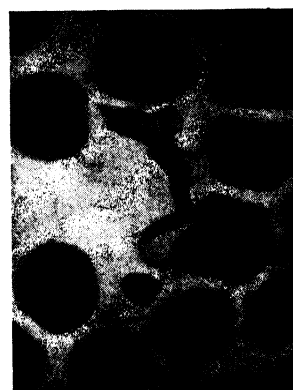


FIG. 22

FIGS. 18-22. Syngamy between a long and an intermediate form. Note migration of the nucleus of one form.

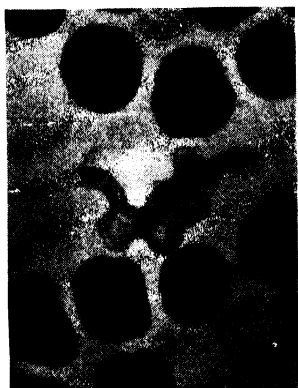


FIG. 23

Two forms with a single nucleus, the flagella arising from different kinetoplasts and being present on either side of the fused mass.



FIG. 24

Note the single kinetoplast and the condition of the nucleus.

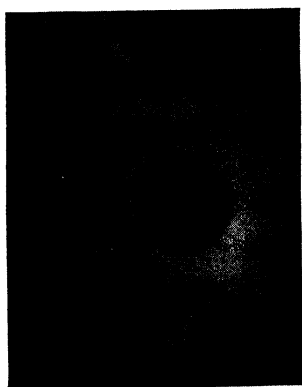


FIG. 25

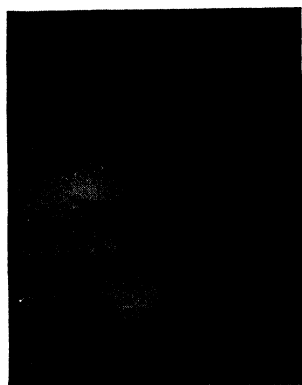


FIG. 26

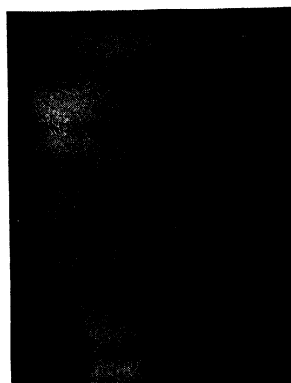


FIG. 27

FIGS. 25-27. Syngamy in *Trypanosoma simiae* in the 'head-to-head' position. The separation of 'ghost' forms of trypanosomes.



FIG. 28

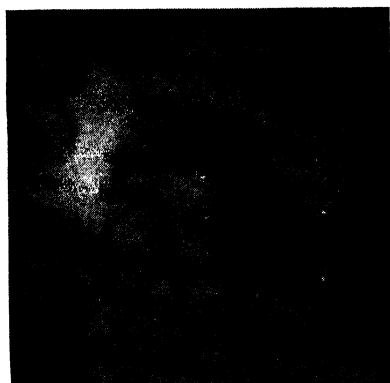


FIG. 29

FIGS. 28-29. Posterior-nuclear forms in the blood of man.



FIG. 30

This illustrates (in a thick film) the severe infection from which the man suffered.



FIG. 31

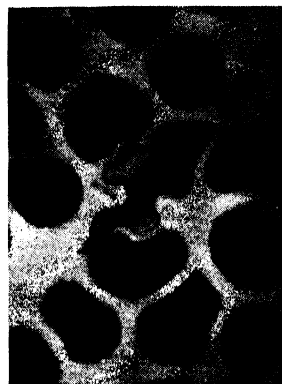


FIG. 32

FIGS. 31-32. *Trypanosoma rhodesiense*. This may be an abnormal type of division in a short form, but it is suggested that it illustrates syngamy in the 'head-to-head' position. Note the single nucleus and single kinetoplast with two flagella in fig. 32.



FIG. 33

Six chromosomes in a short form.

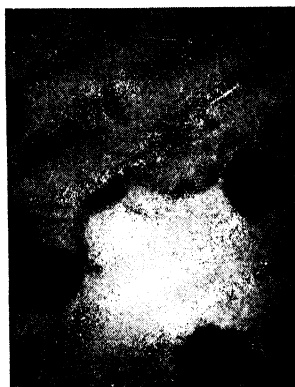


FIG. 34

Six chromosomes in a long form.



FIG. 35



FIG. 36



FIG. 37

FIGS. 35-37. Bodies being extruded from the nuclei. Fig. 35 is *T. rhodesiense* and figs. 36 and 37 *T. simiae*.

THE USE OF AVIAN MALARIA FOR THE DISCOVERY OF DRUGS EFFECTIVE IN THE TREATMENT AND PREVENTION OF HUMAN MALARIA

II.—DRUGS FOR CAUSAL PROPHYLAXIS AND RADICAL CURE OR THE CHEMOTHERAPY OF EXO-ERYTHROCYTIC FORMS

BY

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In 1937 James and Tate announced their discovery, in infections of chicks with *Plasmodium gallinaceum*, of what they called exo-erythrocytic forms of the malarial parasite, forms which inhabited endothelial cells lining the capillaries in various organs of the body and cells of the reticulo-endothelial system, and which lacked pigment because haemoglobin had never been involved in their metabolic processes. For several reasons it was a most important discovery. Admittedly, it was not the first time that malarial parasites had been described in cells other than red cells. Huff and Bloom in 1935 had shown that *P. elongatum* parasitized the whole series of cells between haemacytoblasts and mature erythrocytes. Raffaele, in 1936, had seen large non-pigmented schizonts in the spleen of canaries infected with *P. relictum*, and Kikuth and Mudrow, in 1937, had found them in canaries infected with *P. cathemerium*. But while the activities of *P. elongatum* in parasitizing developmental stages of the erythrocytes, as well as mature stages, might have been dismissed as an interesting oddity, and while the significance of the forms described in canaries may have been only slowly appreciated, because they were few and scattered, and patience was often required to find them, the results of James and Tate were arresting. Their chickens died of a cerebral anoxia caused by a blockage of the capillaries of the brain by strings of comparatively huge schizonts which had developed in the endothelial cells. Similar schizonts were sufficiently plentiful in other organs of the body for them to be found easily.

The abundance of exo-erythrocytic forms in chicks infected with *P. gallinaceum* is itself noteworthy, but James gave great significance to their mere presence. He recalled the work of Raffaele and of Kikuth and Mudrow to show that this was not just a peculiarity of the infections in chicks. He recalled, too, his own work and that of Yorke and Macfie (1924), concerning the differences in the response of sporozoite-induced and blood-induced malarial infections in human beings to treatment with quinine, and his conclusion, announced in 1931, that sporozoites must develop in cells of the reticulo-endothelial system. Quinine can be used to give complete protection to human beings against an infection initiated by the injection of parasitized red cells, but it will not give protection against one initiated by sporozoites, and James argued that clearly if sporozoites penetrated the red corpuscles directly, as Schaudinn believed, there should not be this difference between the therapeutic response of the two types of infection. Hence his belief—a resurrection of a view held by Grassi (1899) for different reasons—that the sporozoites

must develop elsewhere than in the red blood-cells, probably in the reticulo-endothelial cells.

It was obvious that the exo-erythrocytic forms described by James and Tate were not necessarily connected with the development of the sporozoite, for they were present in infections produced by the inoculation of parasitized red cells. But the mere fact that it was now definitely established that the malarial parasite could exist in cells other than red cells made the hypothesis of James concerning the development of the sporozoite much more of a possibility than it had been in 1931.

There was another point concerning exo-erythrocytic forms which James did not overlook. He and Tate (1938) showed that these forms of *P. gallinaceum* were not affected by quinine and that therefore quinine could not be used to cure chicks infected with this parasite. It was well known, of course, that quinine could not be used to cure benign tertian malaria also, although it was very effective against the blood forms of *P. vivax*, and James drew the obvious, even if dangerous, inference.

The impetus given to further research on exo-erythrocytic forms had now become great. The range of species in which they occurred was increased (see the review by Porter and Huff, 1940), and information began to accumulate which showed that sporozoites probably did develop in reticulo-endothelial cells. Missiroli (1937) appears to have seen developmental stages of *P. relictum* in the solid tissues of canaries, and Kikuth and Mudrow (1939b) described such stages existing in reticulo-endothelial cells following the intramuscular injection of sporozoites of *P. cathemerium* into canaries. However, it was not until 1944, when Huff and Coulston published their work on the development of the sporozoites of *P. gallinaceum*, that a reasonably complete account was available of the events between the inoculation of sporozoites and the appearance of parasites in red cells. They demonstrated quite definitely that the sporozoites of *P. gallinaceum* did develop in reticulo-endothelial cells.

It should be noted that all these observations on the presence of exo-erythrocytic forms in malarial infections, whether as part of the cycle of development of the sporozoite or not, have been made in avian malaria, and there is still no definite evidence that they exist in human malaria.

The work of Huff and Coulston was being done, and probably much of it was completed, at a time when a ban existed on the publication of research work on malaria, and they and other workers were forced to proceed along a largely independent course. To those of us engaged in chemotherapeutic research some knowledge of the fate of the sporozoite was essential, and because the available evidence in the literature was by no means unequivocal many preliminary observations had to be made. In these laboratories we had set ourselves three tasks in the chemotherapy of malaria: first, to discover a drug which could be used for clinical treatment and clinical prophylaxis, and which would be without the disadvantages of mepacrine; secondly, to discover a causal prophylactic drug; and, thirdly, to discover a drug to effect radical cures of benign tertian malaria. The general approach to the first problem is obvious, however difficult the details may be. It is concerned entirely with the chemotherapy of the parasites of the erythrocytes, and has been described in the previous paper of this series. The second and third problems, however, demanded the formulation of what could only be hypotheses—facts were too few for the position to be otherwise. Below, in the section headed 'Preliminary Observations,' are given the reasons why the lead of James was followed and why the

hypothesis was formed that the answer to both problems lay in the chemotherapy of exo-erythrocytic forms. In subsequent sections I give a description of the therapeutic tests against exo-erythrocytic forms which were used in these laboratories.

PRELIMINARY OBSERVATIONS

For our purposes I defined a causal prophylactic drug as one which prevented parasites from developing as far as the erythrocytic stages. Such a drug could exert its action on the sporozoites or on the exo-erythrocytic forms into which the sporozoites might develop. It was necessary in the first place, then, to investigate the possibilities of killing the sporozoites, next to satisfy ourselves that exo-erythrocytic stages did precede the erythrocytic parasites, and lastly to determine the period of time before erythrocytic parasites were formed.

There is much evidence that, following the injection of sporozoites into the vertebrate host, a period of time elapses during which the blood is not infective for other animals. This has been shown to be true for infections with *P. falciparum* and *P. vivax* (e.g., Boyd and Stratman-Thomas, 1934; Boyd and Matthews, 1939; Ciuca *et al.*, 1937; Fairley, 1945), *P. cathemerium* (e.g., Warren and Coggeshall, 1937), *P. relictum* (e.g., Raffaele, 1936a) and *P. gallinaceum* (e.g., Henry, 1939). The results which were quoted for the infections with which we were planning to work needed confirmation; e.g., Henry reported a period as long as five days during which the blood of chickens, following their infection by mosquito-bite with *P. gallinaceum*, could not be demonstrated by subinoculation to be infected. Accordingly, experiments were set up to check them.*

Groups of chicks, aged 4-6 days, were injected intravenously with large numbers of sporozoites of *P. gallinaceum* obtained from infected *Aedes aegypti* (in different experiments 5-10 infected mosquitoes were allowed for each chick), and subinoculations were made from them at various times after infection. At least 0.4 ml. of blood—often 0.5 ml.—was withdrawn from each infected chick and injected into a clean chick. It was shown that infective material could be recovered from the blood invariably during a time less than five minutes after the injection of sporozoites, sometimes during a time less than 10 minutes, and not at all during a time longer than 15 minutes and less than 36 hours. The times at which subinoculations were made, if they were later than one hour after infection, were arranged at intervals of six hours, i.e., 6 hours, 12 hours, 18 hours, etc., after infection. Subinoculations between 15 minutes and 30 hours were always negative, but those at 36 hours were almost always positive, and thereafter at least some chicks in a group were always positive. It is well known, of course, that bacteria and viruses—and indeed most finely particulate matter injected intravenously—are rapidly removed from the bloodstream, and that, as infection proceeds, the organisms may return in greater concentration. There is, however, an important difference between this phenomenon as it applies to these organisms and as it applies to the malarial parasite. In the case of bacteria and viruses the duration of the 'negative blood phase' is more variable, depending on the host-parasite relationship and the size of the inoculum; in the case of sporozoites it

* Most of these experiments were done two years ago and more, and references in the text are given only to the published work, or work communicated to me, which influenced them. Recently, Coulston, Cantrell, and Huff (1945) have described their subinoculation experiments, and it should be noted that some of their results are different from mine. The main differences are particularized in footnotes.

appears to have an absolute value for a particular species. That is to say, there is an absolute period of time, following the injection of sporozoites into the host, during which the blood is free from parasites—hence its significance regarding the time and place of development of the sporozoites.

The subinoculation results are so important in relation to the life-cycle of *P. gallinaceum* in the chick that those of one experiment are set out in detail in Table I. For this experiment 12 chicks were heavily infected with sporozoites injected intravenously, and they were then divided into two groups of six each; each chick of the first group was subinoculated by the withdrawal of 0.4 ml. of blood at 30 hours, each of the second group at 36 hours, each of the first again at 42 hours, and so on. This was done because the chicks, at the commencement of the experiment, were only six days old, and it was thought that it was improbable that they would withstand heavy bleeding every six hours. The two groups were therefore bled alternately at 12-hourly intervals, so giving a net effect of subinoculation at intervals of six hours. In the table the sign + or — refers to the development or not of infection in the recipient of the subinoculation.

TABLE I
Subinoculation experiments in chicks following the intravenous inoculation of sporozoites

Chick no.	Hours after infection												
	30	36	42	48	54	60	66	72	78	84	90	96	102
1	—	+	+	+	—	+	+	+	+	+	+	+	+
2	—	+	+	+	—	+	+	+	+	+	+	+	+
3	—	+	+	+	—	—	—	+	+	+	+	+	+
4	—	+	+	+	+	—	—	+	+	+	+	+	+
5	—	—	+	+	+	+	—	+	+	+	+	+	+
6	—	+	+	+	+	—	—	+	+	+	+	+	+
7	—	+	+	+	+	—	—	+	+	+	+	+	+
8	—	+	+	+	+	—	—	+	+	+	+	+	+
9	—	+	+	—	+	—	+	+	+	+	+	+	+
10	—	+	+	+	+	—	+	+	+	+	+	+	+
11	—	+	+	+	+	—	+	+	+	+	+	+	+
12	—	+	+	+	+	—	+	+	+	+	+	+	+
Totals ...	0/6+	5/6+	6/6+	5/6+	4/6+	3/6+	2/6+	6/6+	6/6+	6/6+	6/6+	6/6+	5/5+

The results in Table I suggested the following conclusions:

1. Parasites are not present in the blood of chicks until 36 hours after they have been infected with sporozoites of *P. gallinaceum*. It would seem, taking the results as a whole, that they are continuously present at later times.*

2. The schizogonous cycle of the asexual erythrocytic parasites of *P. gallinaceum* takes 36 hours; the schizogonous cycle of the first generation of the presumed exo-erythrocytic forms also takes 36 hours.

3. At 72 hours all subinoculations are positive and there is an increase in the number of parasites present in the blood. This is reflected in the prepatent periods of the infections in the chicks receiving subinoculations. Previous to 72 hours the prepatent period in the recipient chicks is of the order of 7–12 days, which means that not more than about 10,000 parasitized red cells were injected into them. After 72 hours the prepatent periods

* This result conflicts with the work of Coulston, Cantrell and Huff (1945).

are reduced to 5–6 days and less. The increase in numbers at 72 hours could be due to either or both of two factors, the reproduction of erythrocytic parasites or the reproduction of a second generation of exo-erythrocytic parasites and a further release of young forms into the circulating blood. I favour the view that both factors operate, because parasites are present in the circulating blood continuously between 36 and 72 hours, which suggests that they are erythrocytic forms.

4. Between 42 hours and 72 hours the number of positive subinoculations becomes progressively less. This might be explained by the elimination, during this time, of some of the parasites from the blood-stream by the defensive mechanisms of the host. Their numbers would not be made good until schizogony occurred, at 72 hours, of the erythrocytic and the exo-erythrocytic parasites.

In other experiments which were being done it was sometimes necessary to check the infectivity of the blood in the chicks being studied, and confirmation of the results in Table I were obtained. No chicks subinoculated at 24 hours or 30 hours after infection, 12 of 13 chicks subinoculated at 40 hours, 12 of 20 chicks subinoculated at 48 hours, 3 of 6 chicks subinoculated at 60 hours, and all chicks subinoculated at 72 hours gave positive results in the recipients.

It was now necessary to determine what happened to the sporozoites. The first question to be answered obviously concerned the infectivity of the solid tissues during the 'negative phase' of the blood. Warren and Coggeshall (1937) and Kikuth and Mudrow (1938), working with *P. cathemerium*, had obtained infections in recipient canaries following the parenteral injection of finely ground organs such as the spleen, liver, lung and kidney taken from infected canaries during the time that the blood was non-infective. I was able to do the same with infections of *P. gallinaceum*, but it is an interesting point that a tissue suspension containing the whole of the spleen, or almost all the lung, or half the liver and more, taken from chicks less than 36 hours after they have been heavily infected with sporozoites, does not always produce an infection when injected intraperitoneally into clean chicks. The proportion of failures is much less if the tissues are taken later than 36 hours after the injection of sporozoites. On the other hand, not one failure to infect has followed the implantation, under the wing-skin of a clean chick, of a piece of spleen, usually a quarter or a half of the whole organ, taken from another chick previously injected with sporozoites. Such transplants have been made at many and various times after infection, and success has always been obtained. This procedure, of course, is almost the establishment of a tissue culture *in vivo*, and Hawking (1944, 1945) has demonstrated how easy it is to grow exo-erythrocytic forms of *P. gallinaceum* in tissue culture. In any event, the demonstration that a 'negative phase' in the blood undoubtedly existed following the inoculation of sporozoites, and that infective material could be recovered from tissues such as the spleen during this negative phase, together with the evidence in the literature, was taken to indicate the probability that an exo-erythrocytic development of sporozoites occurred. It remained to find the stages of the development.

The experiments which were done to find them were too crude to give satisfactory results. Very heavy inoculations of sporozoites were given to chicks intravenously—the sporozoites from as many as 50 infected mosquitoes were given to a single chick—and impression smears of the spleens and the lungs were made at various times afterwards and searched for parasites. Only two undoubted parasites, both in the spleen, were seen at times less than 48 hours after infection; one containing four pieces of chromatin was

found at about 30 hours, and the other, a schizont, was seen at about 40 hours. The reason for the failure was clear. Even a million sporozoites, dispersed throughout the body of a chick, would be very difficult to find until reproduction occurred, and from the subinoculation experiments this was thought not to take place for 36 hours. It followed, therefore, that some means of localizing the sporozoites in the chick should be found, but the few attempts which were made to do this were unsuccessful. Huff and Coulston (1944) did succeed in doing so, and they appear to remain the only workers who have seen a complete succession of all the stages in the development of the sporozoite. However, the information which had now been collected—it was the summer of 1943—seemed to justify the view that an exo-erythrocytic phase of development preceded the appearance of parasites in the red blood-corpuscles. The conclusions drawn from these and from other experiments were as follows:

1. The sporozoites, as sporozoites, are too transient in the blood for one to be optimistic about discovering a drug to kill them. Besides, it can be argued that they represent what is essentially a resting stage in the life-history of the parasite. They have completed their development in the mosquito and they are waiting to commence their development in the vertebrate host. Once the development starts they cease to be sporozoites, and until it does start their metabolism is probably at a low ebb. Since most drugs which are now being discovered exert their action by a delicate interference with metabolic processes it would seem that an active metabolism is to be desired as a target.

2. There is a period of 36 hours during which a drug can act on the first generation of exo-erythrocytic forms which follow the introduction of sporozoites of *P. gallinaceum* into chicks. Raffaele (1936a) found a negative blood phase of 65 hours in infections of *P. relictum* in canaries, and Warren and Coggeshall (1937) found one of 72 hours in canaries infected with the sporozoites of *P. cathemerium*. My own experiments have reduced its length in *P. cathemerium* infections in canaries to 48 hours. In human malaria the duration of the negative blood phase is several days (Boyd and Stratman-Thomas, 1934; Boyd and Matthews, 1939; Ciuca *et al.*, 1937; Fairley, 1945).

3. At the end of the initial negative phase of the blood, parasites appear to be continuously present in it, and, in the case of *P. gallinaceum*, their number increases at 72 hours. The exo-erythrocytic forms also increase in numbers and they persist as long as infection lasts with *P. gallinaceum*, *P. cathemerium* and *P. relictum*. The results of some early experiments suggested that the later exo-erythrocytic forms reacted differently from the earlier ones to drugs, and accordingly a classification into primary and secondary exo-erythrocytic forms was adopted. However, further work made a full interpretation of all the results very difficult, as will be seen from the experiments with Paludrine which are described below.

4. Neither mepacrine* nor quinine affected the development of either the early or the later generations of the exo-erythrocytic forms of *P. gallinaceum*, *P. cathemerium* or *P. relictum*, and consequently they could not be used either to protect or to cure. Pamaquin,† on the other hand, was claimed to have an action on the exo-erythrocytic forms of *P. cathemerium* (Kikuth and Mudrow, 1939a), a result which I confirmed (see below), and it also has a causal prophylactic action in human malaria and helps to lower the relapse-rate of

* Synonyms are atabrine, atabrin and quinacrine.

† Synonymous with plasmochin and plasmoquine.

benign tertian malaria. The analogies between the types of avian malaria which were being used in these laboratories and benign tertian malaria therefore appeared quite striking—in fact, the only difference was that exo-erythrocytic forms had not been seen in cases of benign tertian malaria. At any rate, the analogies were sufficiently close for the decision to be made to give the chemotherapy of exo-erythrocytic forms as much attention as that of the blood forms.

CHEMOTHERAPEUTIC TESTS AGAINST EXO-ERYTHROCYTIC FORMS

I. TESTS AGAINST THE EXO-ERYTHROCYTIC FORMS OF *P. gallinaceum*

Brumpt (1936) showed that *P. gallinaceum* is successfully transmitted by *Aedes aegypti*. Because of the ease with which this mosquito is bred in the laboratory, the readiness with which the females gorge, and the usually large numbers of sporozoites which develop in them, it was used for all the experiments in which a sporozoite-induced infection of *P. gallinaceum* was desired. Particulars concerning the establishment of the infections, the treatment accorded them, and the results obtained, are given below.

1. *Experimental Animals*. Unless otherwise stated, these were six-day-old chicks, weighing 45–55 gm., and similar to those previously described (Davey, 1946).

2. *The Inoculum*. In the beginning the salivary glands were dissected from infected mosquitoes which had been immobilized by a very short—less than 30 seconds—exposure to ether. There are reports in the literature that ether is deleterious to sporozoites, and there is no doubt that it is so when they are exposed to its effects for a matter of minutes. However, I have never had reason to think that the very short exposure which has been given in this laboratory is unsatisfactory for the purposes of these experiments. When the glands were removed from the mosquito they were added to a mixture of equal parts of heparinized chick plasma and Ringer-Locke solution and were broken up. An attempt was always made to have sufficient mosquitoes to allow approximately one pair of infected glands for each experimental chick. The broken glands were pooled and diluted with sufficient plasma mixture to allow 0.2 ml. for each chick.

Later, as the experiments increased in size and number, this method was forsaken, and the entire mosquitoes were simply ground in a pestle and mortar in a small quantity of heparinized chick blood. This was then diluted with Ringer-Locke and lightly centrifuged on a hand centrifuge to remove the heavy chitinous parts and larger pieces which had resisted grinding. About 20–30 turns were found sufficient for this purpose. The supernatant, diluted to give the concentration, roughly, of one mosquito per 0.2 ml., was used as the inoculum. Still later this method, too, was discarded, and all that is now done is to grind the mosquitoes in a Griffiths tube. A few minutes' grinding is sufficient to reduce all their parts to a sufficiently small size to allow the suspension to be given intravenously to chicks.

3. *The Route of Inoculation*. An experiment was done to determine the best method of infecting the chicks. Too many were being used for this to be accomplished by the bite of mosquitoes, and in any event it was desired to standardize the infection more than such a procedure would allow. An inoculum, prepared as usual to allow 0.2 ml. to contain the contents of approximately one infected mosquito, was distributed amongst four groups of chicks, each group containing six chicks. One group received it intravenously, the second intramuscularly, the third intraperitoneally, and the fourth subcutaneously. The

results are shown in Table II. In this table the + sign signifies parasites in blood smears and the description 'dead' implies that the chicks died from massive infections with exo-erythrocytic forms.

It will be seen that intravenous inoculation is the method of choice, although intramuscular inoculation is almost as good.* In all our experiments, therefore, intravenous inoculation of sporozoites was adopted as the standard procedure. Many thousands of chicks have been infected in this way, and it can be taken that the method is certain and the results constant. I am unable to explain why some workers have found it unsatisfactory.

TABLE II

Differences in the course of infection of *P. gallinaceum* in chicks according to the route of inoculation of sporozoites

Route of inoculation	Days of experiment : inoculation on day 1										
	7	8	9	10	11	12	13	14	15	16	
Intravenous ...	6/6+	6/6 dead									
Intramuscular	1/6+	6/6+		3/6 dead	6/6 dead						
Intraperitoneal	6/6—	4/6+	6/6+ 1/6 dead			3/6 dead		4/6 dead	5/6 dead		Remaining one killed 20th day
Subcutaneous	6/6—	1/6+	3/6+	6/6+	1/6 dead	2/6 dead			3/6 dead	4/6 dead	Remaining two killed 20th day

4. *The Viability of the Sporozoites in the Inoculum.* For some of the experiments as many as 150 chicks were used. Their inoculation, with three workers injecting, should not normally take more than about half an hour, and the inoculum, including the time taken for its preparation, would not then be more than three-quarters of an hour old when the last chick is injected. However, circumstances sometimes caused more time to be taken for these purposes, and it was necessary, therefore, that the viability of the sporozoites in inocula of different ages should be investigated. The results are given in Table III.

It will be seen that, although the sporozoites in the inoculum survive better at low temperatures, yet survival at laboratory-temperatures is sufficiently good for most experiments, and it is only in exceptional circumstances that the inoculum need be kept in an ice-bath. Brackett and Hughes (1945) keep the sporozoites chilled as a routine procedure, but their experiments are so big that inoculation takes them at least an hour.

5. *The Course of Infection in the Standard Experiments Used for Testing Drugs.* The procedure in these experiments was to allow, if possible, about one infected mosquito for each chick, to prepare a pooled inoculum as described above, and to inject it intravenously. Methods of counting sporozoites in suspensions have been described (e.g., Shute, 1937), but sporozoites tend to clump together and to stick to surfaces in such a degree that the accuracy of the counts is very open to doubt. The standardization adopted here may also appear of doubtful value, because the implication seems to be made that one infected mosquito is much like another; but actually, remembering the size of the experiments, what is implied is that one hundred or so infected mosquitoes are much like another

* With smaller inocula, the intravenously injected birds not dying until the 10th day, the differences in Table II are more accentuated.

hundred or so, which is probably true. However, success in standardization of the infection is best measured by its course, and this undoubtedly can be made very prescribed.

It will have been apparent from Tables II and III that parasites can be seen in blood smears of the chicks taken on the 7th day of the infection. They are found easily at this time, with more difficulty on the 6th day, and only after quite prolonged search on the 5th day. Since a drug which has an action on the exo-erythrocytic forms will either prevent or delay the appearance of parasites in the blood, the examination of smears can be used, in part, to assess the efficacy of a treatment. It might be thought that a drug very active against blood forms could give a similar picture, but it seems that it will be a very exceptional drug which will do so. The reservoir which the exo-erythrocytic forms constitute is an active one which is almost continuously releasing erythrocytic forms. The reproduction of the latter in the blood-stream is inhibited by the usual antimalarial drugs, but their entry into it is not, and so it happens that in infections treated with mepacrine or quinine parasites, albeit degenerate ones, can still be detected in the red cells.

TABLE III

Course of infection of *P. gallinaceum* in chicks following the intravenous injection of sporozoites kept in a mixture of heparinized whole blood and Ringer-Locke for various times and at various temperatures

Particulars of inoculum	Days of experiment : inoculation on day 1							
	7	8	9	10	11	12	13	14
Kept $\frac{1}{2}$ hour at laboratory-temperature (22° C.)	6/6 + 2/6 dead	6/6 dead						
" 1 " " " ...	6/6 +	4/6 dead	6/6 dead					
" 2 hours " " ...	6/6 +	2/6 dead	6/6 dead					
" 3 " " " ...	6/6 +	3/6 dead	6/6 dead					
" 4 " " " ...	6/6 +	1/6 dead	4/6 dead	6/6 dead				
" 24 " " " ...	No infection							
" 3 " 37.5° C. ...	6/6—	6/6 +			3/6 dead	5/6 dead		6/6 dead
" 24 " 3° C. ...	4/6 +	6/6 +		3/6 dead	5/6 dead			6/6 dead

However, there is another criterion of activity which is more unequivocal in its interpretation. The exo-erythrocytic forms, which are found with such great difficulty in the early stages, are capable of so rapid an increase in numbers that by the 7th day or a little later the capillaries of the brain may become blocked with them, and the chicks die of a cerebral anoxia. This event occurs with complete certainty if the inoculum is sufficiently big for the infection to become well established before the age of the chick is such that its immunity responses interfere appreciably with the increase in numbers of the parasites. It occurs, too, over a narrow range of time—usually 3–4 days. This point is illustrated in Table IV, in which aggregate results, drawn from many experiments performed during the past two to three years, are summarized. The experiments were classified according as the controls commenced to die on the 7th day or the 8th day, and so on. The number of daily deaths on this and subsequent days is expressed in the table as a percentage of the total number of chicks in the group.

The arrangement in Table IV implies that there has been a variation in the inoculum or in the chicks from time to time, which is at least true of the inoculum. It was stated above that an effort was made to allow one infected mosquito for each chick in all experiments; but sometimes the numbers of mosquitoes were insufficient for this and the inoculum had to be reduced. The results in Table IV show, however, that, even if the chicks do not commence to die until the 10th day, the mortality curve is still so steep that an experiment is easily interpreted and therefore well suffices for initial tests. It is not until the strength of the inoculum is reduced so low that the chicks do not commence to die until about the 12th day that an experiment may become difficult to read because of the spread of the mortality curve.

TABLE IV

The mortality-rate in chicks infected with sporozoites of *P. gallinaceum*, the chicks being grouped according to the day on which deaths commenced in the different experiments

Group	Percentage deaths on indicated day of experiment								
	7	8	9	10	11	12	13	14	15
I	27.3	63.6	9.1						
II		44.5	43.1	8.2	4.15	0.05			
III			41.8	42.5	10.9	2.1	2.1	0.6	
IV				51.8	31.3	13.2	3.7		
V					25	18.8	32.2	12.5	12.5

6. *Treatment.* From what has been said above it will be realized that any treatment which saves the life of a chick infected with sporozoites of *P. gallinaceum* must affect either the sporozoites themselves or the exo-erythrocytic forms. Mepacrine and quinine lack both capacities, and it follows that treatment with either, however long and intensely it is made within the limits imposed by their toxicity, does not influence the mortality-rate. In the beginning, then, in the search for drugs for causal prophylaxis and radical cure, the exact duration of treatment is merely a matter of convenience. No concern need be felt about the phase which is being acted upon, sporozoite, earlier exo-erythrocytic forms, or later exo-erythrocytic forms, because it will be an achievement to discover a substance acting on any of them. Once such a substance is found—that is to say, once the mortality-rate is being influenced—the details of its action can be investigated later.

In this laboratory, therefore, treatment of the infection has been carried on as long as is conveniently possible. It has been usual to infect the chicks on Monday. They are sorted into groups and given the first dose of the test substances about two hours before infection; all substances, unless otherwise stated, have been administered orally as described in the previous paper (Davey, 1946). The purpose of the first dose is to allow the substances under test to be in the blood-stream at the time when the sporozoites are injected. The second dose is given three to four hours after infection, and the treatment is then repeated twice daily on each of the next five days. It finishes, therefore, only one day before deaths amongst untreated birds are to be expected, and clearly a substance is given every opportunity of exerting any action it may have against exo-erythrocytic forms.

7. *Results with Mepacrine, Pamaquin, Sulphonamides* and Paludrine.* Some of the

* Information concerning the action of sulphonamides (sulphadiazine) in this infection was first obtained from the Board for the Co-ordination of Malarial Studies, U.S.A., in 1943; the information has since been published (Coatney and Cooper, 1944; Coggeshall, Porter and Laird, 1944):

results which have been obtained with these substances are summarized in Table V and others are discussed in subsequent paragraphs. It should be noted that when cure is claimed the result has been checked by subinoculation. Three to four weeks are allowed to elapse following the cessation of treatment, and during this time blood examinations are done. If these are negative, subinoculations of as much as 1 ml. of blood are made, and the recipient chicks are observed for the next three weeks.

TABLE V
Activity of substances against infections in chicks with sporozoites of *P. gallinaceum*; treatment commenced in all cases on the day of infection

Substance	Treatment	Results
Mepacrine hydrochloride	8 mgm. b.i.d. as long as possible	No apparent effect on mortality-rate.
Sulphadiazine ...	25 mgm. b.i.d. × 4	Cure.
	12.5 " " × 4	Some cures.
	6.25 " " × 4	Marked delay in course of infection; no cures.
	1 " " × 4	" " " " in some chicks.
	0.5 " " × 4	No apparent effect on mortality-rate.
Sulphamezathine	25 " " × 4	Cure in most chicks.
	12.5 " " × 4	Marked delay in course of infection: no cures.
	4 " " × 4	" " " " " " " "
	2 " " × 4	Death "delayed some " days in a " few chicks. "
Sulphamerazine	25 " " × 4	Marked delay in course of infection; no cures.
Sulphanilamide	25 " " × 4	" " " " in all chicks; a few apparent cures.
Sulphathiazole ...	25 " " × 4	Marked delay in course of infection; no cures.
Sulphapyridine	25 " " × 4	" " " " " " " "
Pamaquin (dis-naphthoate)	2 " " × 6	Death "delayed a few " days; cure " not attained.
	1 " " × 6	No apparent effect on mortality-rate.
Paludrine hydrochloride	3 " " × 5*	Cure.
	2 " " × 5	Some cures.
	1 " " × 5	Marked delay in the course of infection.
	0.25 " " × 6	Death delayed some days.
	0.1 " " × 6	" " " " in some chicks.

* This treatment is toxic to some chicks.

Other results obtained with Paludrine, sulphonamides and pamaquin are as follows:

1. The curative effect, or the influence on the mortality-rate, exerted by these substances is due in every instance to an action on the exo-erythrocytic forms; none of them has an action on sporozoites. This can be shown in three ways. Firstly, treatment can be delayed until after the sporozoites have disappeared from the circulating blood and the same results can still be achieved. Secondly, treatment concentrated at the time when the sporozoites are injected has only a partial effect at the most; it must be continued for some days to achieve full effects. Thirdly, sporozoites may be exposed to big concentrations of the drugs *in vitro* without killing them; e.g., sporozoites which had been exposed for one hour to concentrations of 5 mgm./litre of sulphadiazine or Paludrine in a mixture of equal parts of chick plasma and Ringer-Locke kept at 39° C. were still capable of infecting chicks.

2. Paludrine exerts its action against exo-erythrocytic forms much more rapidly than the sulphonamides. Thus, if chicks are injected with the 'normal' inoculum of sporozoites, so that their death may be expected about the 8th day, treatment with sulphadiazine commenced on the 5th day may not even save their lives. Treatment with

Paludrine, on the other hand, may be started when the chicks are almost moribund and their death may still be prevented.

The poor results obtained with sulphonamides when they are used late in the infection was one of the reasons why it was thought that there might be primary and secondary exo-erythrocytic forms which reacted to drugs differently. However, as more experiments were done it became clear that the details of this result with the sulphonamides could be altered at will by varying the number of sporozoites used for the infection, and therefore its explanation is bound up simply with the rate of action of these drugs.

3. A distinction between primary and secondary exo-erythrocytic forms is still envisaged as a possibility because of certain other results which, as yet, cannot be explained. Paludrine, in a dose of 3 mgm. per 50 gm. chick administered twice daily for five days, can be used to cure all chicks injected with sporozoites, provided that treatment does not commence later than about 48 hours after the time of inoculation, and it will cure many if treatment does not commence later than about 80 hours afterwards; but when treatment has been delayed for four days no cures have been achieved. The infection can still be controlled easily at these later times, but sterilization of it does not seem to be possible with Paludrine. Similar results have been obtained with sulphadiazine and sulphamezathine. The experiments using Paludrine in a dose of 3 mgm. per 50 gm. chick given twice daily for five days must be planned on a big scale initially because of the delayed deaths that occur with this treatment. Usually, about one-third of the birds die, but sometimes more than half of them may do so.

Until it is fully understood why the infection becomes progressively more difficult to cure, it seems wiser to err on the side of safety and to take into account the fact that later generations of exo-erythrocytic forms may be different from earlier ones. The later generations are co-existent in the host with parasites in the red blood-corpuscles and may include forms derived from them. The complex conditions which then exist are like those which follow an inoculation of parasitized red cells into chicks; both the main forms of the parasite, erythrocytic and exo-erythrocytic, become established, and maybe it is significant that Paludrine cannot be used to sterilize this infection either.

It is possible to think of reasons why later generations of exo-erythrocytic forms may be different from earlier ones. The metabolism of the parasite may be related to the type of cell it inhabits—it certainly seems to be related to the presence or absence of haemoglobin in a cell—and it is, perhaps, an important point that not only do the numbers of exo-erythrocytic forms increase during the course of the infection, but a wider variety of cells becomes parasitized. In the beginning the cells which are invaded belong to the reticulo-endothelial system; later, endothelial cells are also invaded. Porter (1942) has observed this in canaries infected with *P. cathemerium*, and it is equally true of *P. galinaceum* in chicks. Endothelial cells in the brain capillaries, for example, become parasitized only late in the infection—I have not seen them earlier than 4½ days after inoculation—whereas reticulo-endothelial cells are parasitized from the commencement (Huff and Coulston, 1944). Again, two different types of schizonts have been described, those with macromerozoites and others with micromerozoites, and it appears that 'there is . . . a shift in successive generations from the former to the latter type' (Huff and Coulston, 1944). However, while such reasons should be borne in mind, other evidence can be produced to make it doubtful if they provide the whole answer (see below).

Experiments are continuing in an attempt to elucidate the problem ; in the meantime the observations which have been made are summarized in the accompanying chart (p. 464).

4. It will be apparent from Table V that with both Paludrine and sulphadiazine there is a wide range of doses over which an action on the exo-erythrocytic forms can be demonstrated. The high doses, given for a comparatively short time, cure, and it was thought that the same effect might be produced by giving lower doses for a longer time, but many experiments were made with Paludrine, using doses varying between 0.5 mgm. and 1.5 mgm./50 gm., administered twice daily for as long as a month, without achieving this end. It was then decided to find the minimum dose required to inhibit reproduction of the exo-erythrocytic forms before continuing with such protracted treatments, and the interesting result was obtained that the minimum inhibiting dose is also the minimum curative dose. In these experiments the chicks were infected with sporozoites and treatment started on the first day. Subinoculations (0.5 ml. blood) were made from them on the 8th day, the treatment being continued until then, and it was argued that when the subinoculations failed to reveal parasites in the circulating blood the reproduction of the exo-erythrocytic forms was inhibited. The minimum dose which achieved this, although not in all chicks, was 2 mgm./50 gm. given twice daily, which is also the lowest dose with which cures of the sporozoite-induced infection have been obtained.

Similar subinoculation experiments were done with chicks infected by means of parasitized red cells. Such chicks were treated twice daily with varying doses of different drugs, and subinoculations (0.5 ml. blood) were made at different times while treatment was still being continued. No subinoculation was made earlier than the 7th day after the commencement of treatment, so that all drugs received ample opportunity for exerting their full effect. Subinoculations gave positive results with mepacrine (10 mgm./50 gm. b.i.d.), sulphadiazine (50 mgm./50 gm. b.i.d.) and pamaquin (2 mgm./50 gm. b.i.d.), but negative results were obtained with Paludrine (3 mgm./50 gm. b.i.d.); some negative results were also obtained with Paludrine at the lower dose of 2 mgm./50 gm. Several interesting facts emerged from these experiments :

(a) It would be expected that such high doses of mepacrine and pamaquin, since they are very good schizonticidal drugs, would eliminate the parasites of the red cells as such, and I therefore interpret the continued infectivity of the blood in birds being heavily and continuously treated with these drugs as being due to the release of parasites from the reservoir of infection constituted by the exo-erythrocytic forms.

(b) The comparative pooriness of the action of sulphadiazine against exo-erythrocytic forms in a well-established infection is noteworthy.

(c) Treatment with 0.1 mgm. b.i.d. of Paludrine has some influence on exo-erythrocytic forms (Table V). Increasing the treatment twenty-fold does not even lead to complete inhibition of their reproduction in all chicks, and increasing it thirty-fold, although leading to cure of early infections with sporozoites, and sterilization of the circulating blood in all infections, only inhibits reproduction of exo-erythrocytic forms in well-established infections or infections initiated by erythrocytic forms.

(d) Apparently complete inhibition of reproduction of the exo-erythrocytic forms in a blood-induced infection has been maintained with Paludrine for as long as 17 days without sterilizing the infection.

(e) The number of parasites surviving in the tissues of chicks receiving 3 mgm./50 gm. b.i.d. of Paludrine appears very small, and they are recovered with difficulty. Their

number is so small that it is hard to believe they are characterized only by inhabiting a particular type of cell, or that they represent a major form of the parasite, such as schizonts with micromerozoites. Besides, no evidence has yet been obtained that the parasites which have been recovered from the tissues (kidney, spleen) in these experiments represent a resistant type of exo-erythrocytic form. Details of these experiments will be published later.

5. The slight action which pamaquin possesses against exo-erythrocytic forms which was indicated in Table V can be confirmed in another way. When six-day-old chicks are injected with some millions (20–50) of parasitized corpuscles they die in about a week from the acute parasitaemia. If they are continually treated with mepacrine or quinine the acute parasitaemia is prevented, but they survive only to about the 16th or 20th day, when they die from a massive exo-erythrocytic infection. Sulphonamides and Paludrine, of course, prevent this second event, and so, too, does pamaquin if it is given in high doses (2 mgm./50 gm. b.i.d.). Such doses are toxic and deaths from poisoning may occur about the 25th day of the experiment. However, treatment can be continued sufficiently long to show quite definitely that pamaquin has an action on exo-erythrocytic forms. The results with smaller treatments showed that 1.5 mgm./50 gm. b.i.d. had some action in some chicks but that 1.0 mgm. b.i.d. apparently had none. Coggeshall, Porter and Laird (1944) have also reported a slight action of pamaquin against these forms, so slight that they thought, sometimes, that it was questionable.

II. TESTS AGAINST THE EXO-ERYTHROCYTIC FORMS OF *P. cathemerium* AND *P. relictum*

Because of the war, canaries could not be obtained in sufficient numbers for all the preliminary experiments with *P. cathemerium* and *P. relictum* that were desirable, and certain of the results obtained from the experiments with *P. gallinaceum* were transferred without check to these other experiments. The inoculum has been prepared in the same way, except that only the thoraces of the mosquitoes (*Culex molestus**) have been ground, and it has been injected intravenously. Half to one infected mosquito has been allowed for each canary on experiment.

Sporozoites apparently disappear from the blood of canaries infected in this way with *P. cathemerium* within 15 minutes of injection, and parasites have not been recovered in subinoculation experiments until 48 hours afterwards; too few canaries have been used for this to be quoted as the minimum duration of the 'negative blood phase.' Parasites may be found in blood smears, about one in 100 fields, 96 hours after infection, and thereafter their number progressively increases. Most of the birds infected with the M. strain of *P. cathemerium* die from the acute blood infection. Those infected with the Algerian strain of *P. relictum* attain control of the parasites and the infection lapses into a chronic state.

Exo-erythrocytic forms are found easily in impression smears of the spleen taken at the time when parasites are found in blood smears, but there is no evidence that their number is so many that they contribute to the death of the host. However, the reservoir of exo-erythrocytic forms is big enough to release sufficient parasites into the circulating blood for parasitized red cells to be found easily, even in birds continuously treated at

* I am indebted to Dr. Ann Bishop for supplying me with the strain of mosquito and also for the Algerian strain of *P. relictum* which was used in some of the experiments.

maximum tolerated doses with mepacrine and other drugs (e.g., 4316, sontoquin) which have a marked action on the blood parasites. The parasites which are found are degenerate but unmistakable.

The initial test of a substance for activity against the exo-erythrocytic forms of *P. cathemerium* is therefore easy. The birds which have been infected with sporozoites are treated twice daily from the beginning of the experiment with the appropriate solution or suspension—all the canaries in these experiments were dosed orally—until the controls exhibit parasites in the blood. In all the experiments I have done the only drugs which have prevented parasites from appearing in the treated birds at the same time as they appeared in the controls are pamaquin and Paludrine and compounds related to them. With both pamaquin and Paludrine, however, parasites appear three or four days after the cessation of treatment. The result with pamaquin is substantially the same as that reported by Tate and Vincent (1933) for *P. relictum*, except that the variation amongst their controls, because they were infected by direct mosquito-bite, was too great for the significance of the delay to be apparent.

The delay in the appearance of parasites associated with treatment with these two drugs can only be due to their action on exo-erythrocytic forms. Even if they were amazingly rapid in acting on parasites within the red cells, the parasites should still be visible as degenerate forms. In any event, if smears are made from the spleen of the canaries under treatment with them, exo-erythrocytic forms are not found so easily as they are in smears made from canaries serving as controls or treated with mepacrine. Of course, it is difficult to give a strict value to the density of these forms in the spleen, but that there is a smaller number present in birds treated with Paludrine and pamaquin is undoubted. Kikuth and Mudrow (1939a) also believe that pamaquin (plasmoquine) has an action on the exo-erythrocytic forms of *P. cathemerium*.

The action of both pamaquin and Paludrine against the exo-erythrocytic forms is only weak. The reproduction of some of them is inhibited, so that fewer merozoites are released to invade red cells. The fact that not all of them are inhibited in this way can be shown by subinoculation experiments from canaries infected with sporozoites and which are continuously treated with maximum tolerated doses of Paludrine (1.5 mgm./20 gm. b.i.d.) or pamaquin (0.2 mgm./20 gm. b.i.d.). Blood smears continue to be apparently negative in such birds but the subinoculations are positive. Treatment has been continued for as long as nine days after infection and still the subinoculations, on the last day of treatment, yield positive results.

The results described above have, unless a direct reference is made to *P. relictum*, been obtained from experiments with *P. cathemerium*. It can be taken, however, that the course of infection with *P. relictum* (Algerian strain) is very similar, and I have not yet observed any difference between the two species in their reactions to pamaquin or Paludrine.

III. CAUSAL PROPHYLACTIC EXPERIMENTS WITH *P. lophurae*

Three species of hosts, six-day-old chicks and ducklings and mature canaries, were used in attempts to establish a causal prophylactic test against *P. lophurae*, but none of them was satisfactory. The infection in each is much too benign, and of much too low a grade, for results to be easily interpreted. These facts, particularly in relation to chicks and ducklings, are most interesting. Both species are very satisfactory hosts for the parasites of the red blood-corpuscles. The parasitaemia climbs quite high in the chick

and extraordinarily high in the duckling—3-4 parasites may be found in almost every corpuscle—following the intravenous inoculation of parasitized red cells. But in both these hosts the intravenous inoculation of even massive quantities of sporozoites—the vector in these experiments was *Aedes albopictus*—gives rise to only scanty infections (see also Laird, 1941). The prepatent periods are long, and when parasites do appear only small numbers are found. Some typical results for untreated birds are given in Table VI.

TABLE VI

Course of infection in canaries, chicks and ducklings following the intravenous inoculation of large numbers of sporozoites of *P. lophurae*; the density of the infection is given as parasites per field

Host	Days of experiment : inoculation on day 1											
	1-6	7	8	9	10	11	12	13	14	15	16	17
Canary ...				—	1/50	1/5	1/5	1/50 1/2	1/10 1/1	1/5 2/1	1/2 1/10	1/2 1/50
Chick ...		— — —	1/100 — —	1/20 — —	1/20 1/100 —	1/50 1/50 1/100	1/50 1/20 1/100	— 1/5 1/50	1/50 1/20	— 1/5	1/5	1/50
Duckling			1/100 1/100 —	1/50 1/50 —	1/50 1/10 1/100	— 1/5 —	— 1/5 1/100	— 1/50 1/50	— 1/20			

It will be clear that causal prophylactic experiments with such infections cannot be made satisfactorily. Negative results, i.e., parasites present in treated birds, are valuable, but it is difficult to be certain of the true meaning of what appear positive results, and it would be rash to attach any significance to what might seem a delay in the appearance of parasites. The results recorded below are the most important which I have obtained. In all the experiments the first dose was given two hours before infection, the next about four hours after infection, and treatment was repeated twice daily on each of the next three days.

(a) Mepacrine (8 mgm./50 gm. b.i.d. \times 4) does not prevent parasites from appearing in the blood of chicks.

(b) Sulphamezathine (25 mgm./50 gm. b.i.d. \times 4) is similarly inactive in chicks and ducklings.

(c) Paludrine at 3 mgm./50 gm. b.i.d. \times 4 appears to have completely protected chicks; it failed at 2 mgm./50 gm. b.i.d. \times 4.

(d) Paludrine at 2 mgm./20 gm. b.i.d. \times 4 appeared completely to protect two of three canaries; it failed at 1 mgm./20 gm. b.i.d. \times 4.

P. lophurae was obtained originally from a Borneo pheasant (Coggeshall, 1938), and it was hoped that English pheasants could have been used for some experiments, but arrangements could not be made to obtain them in sufficient numbers. Also, arrangements could not be made to obtain turkey poults in quantity, although it was learnt that Porter* had shown that in these animals the course of a sporozoite-induced infection with *P. lophurae* is very like that of a sporozoite-induced infection with *P. gallinaceum* in chicks; a similar massive development of exo-erythrocytic forms occurs from which the birds die.

* Communication from the Board for the Co-ordination of Malarial Studies, U.S.A.

SUMMARY

1. Reasons are given why it is thought that the problems of discovering drugs for causal prophylaxis and radical cure are linked with the chemotherapy of exo-erythrocytic forms. A drug which kills sporozoites would also be a causal prophylactic drug, but it is pointed out that such a drug may be very difficult indeed to find.

2. Some observations on the life-cycle of *P. gallinaceum* in the chick included the demonstration that, following the inoculation of sporozoites, parasites appear in the blood 36 hours later. They are continuously present afterwards, and it is suggested, therefore, that they are erythrocytic forms.

3. During the 'negative blood phase' parasites can be recovered from the solid tissues. This is done most surely by implanting a piece of spleen taken from an infected chick under the wing-skin of a clean chick. It can also be accomplished in a fair proportion of cases by injecting intraperitoneally the finely ground tissues.

4. Parasites have been recovered by subinoculation from canaries 48 hours after they were injected with sporozoites of *P. cathemerium*.

5. The course of infection of *P. gallinaceum* in six-day-old chicks following the intravenous injection of sporozoites is described, together with the use of the infection in testing substances for an action against exo-erythrocytic forms. The use of *P. cathemerium* and *P. relictum* in canaries for a similar purpose is also described. It is demonstrated that a sporozoite-induced infection of *P. lophurae* in chicks, ducklings and canaries leads to infections which are much too benign, and of too low a grade, for them to be generally satisfactory in chemotherapeutic experiments.

6. The most important chemotherapeutic results which have been obtained are as follows:

(a) Mepacrine and quinine have not been demonstrated to have any action against any exo-erythrocytic forms.

(b) Paludrine can be used to protect chicks completely against infection with *P. gallinaceum*, provided that treatment does not commence later than about four days after the inoculation of sporozoites. At later times it still controls the infection easily, but cure has not been obtained. Similarly, cure has not been obtained of blood-induced infections, even though the blood-stream has been kept free of parasites and the reproduction of the exo-erythrocytic forms kept inhibited, seemingly completely, for as long as 17 days. It is not known why the infection becomes progressively more difficult to cure, and therefore the possibility that later generations of exo-erythrocytic forms react differently to drugs cannot be ignored.

(c) Paludrine appears to prevent infection in chicks and canaries inoculated with sporozoites of *P. lophurae*.

(d) Its action against the exo-erythrocytic forms of *P. cathemerium* is only mildly inhibitory and is paralleled by that of pamaquin. Pamaquin also has a slight action against these forms of *P. gallinaceum*.

(e) Sulphonamides have a marked action against the exo-erythrocytic forms of *P. gallinaceum* but none against those of *P. cathemerium*. They act more slowly than Paludrine.

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BLOOD AND PLASMA CONCENTRATIONS OF MEPACRINE IN SUBJECTS TAKING SUPPRESSIVE AND THERAPEUTIC DOSAGES

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The work described in this paper was carried out during 1943-44. For convenience, the subject-matter has been divided into separate sections, which are treated independently.

I. THE CONCENTRATIONS ATTAINED AFTER A SINGLE DOSE OF 100 MGM. MEPACRINE

After the oral administration of a single dose of 100 mgm. mepacrine hydrochloride to a fasting subject who has not previously had the drug, mepacrine becomes detectable in the plasma in some cases within an hour of ingestion, and in all cases at some time between the 2nd and 4th hour after dosage. If estimations of plasma mepacrine content are made hourly after a single dose of 100 mgm., it is possible to plot the figures in the form of a curve, which we have termed the 'post-absorption curve' and which can be taken to represent at any one point the balance between the introduction of the drug into the plasma as a result of absorption and the removal of the drug from the plasma as the result of fixation in blood-cells and tissues and excretion in the urine. The post-absorption curve in most subjects reaches its peak 2-4 hours after dosage. The degree of rise at the peak period and the general shape of the curve appear to be independent of the plasma concentration obtaining at the time of administration of the dosage. This can be seen in fig. 1, in which the post-absorption curves on the 1st and 7th days of administration of a suppressive course of 100 mgm. daily (nine subjects) are compared with similar curves on the 58th day of dosage (four subjects). We take this to indicate that absorption from the gut continued at approximately the same rate on the 7th and 58th days of the course as at the beginning of the dosage régime. It will be noted from inspection of fig. 1 that the whole-blood post-absorption curves on the 1st, 7th and 58th days are all of different orders, the curve on the 58th day showing the greatest peak rise. For reasons stated elsewhere (Army Malaria Research Unit, 1946a) we consider the plasma concentrations to be of greater physiological significance than those of the whole blood.

In any individual subject we have found that the plasma post-absorption curve following a dose of 100 mgm. mepacrine varies little, provided that the conditions of administration are kept constant. It is possible, however, by various means, such as the administration of food, to alter the shape of the curve (which we take to be equivalent to altering the *rate* of absorption). We have not been able to discover any means whereby the total absorption over 24 hours can be affected. The significance of these findings is discussed elsewhere (Army Malaria Research Unit, 1946c).

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One point of practical importance in experimental work on mepacrine is obvious. In view of the daily variations of blood and plasma concentrations following dosage, it is essential to fix a standard time for the withdrawal of blood samples. This time of withdrawal should be constant for the whole group under observation. As will be seen from fig. 1, this standard time is more important in the case of whole-blood concentrations than of plasma. Thus, two hours after administration of the dose in the four subjects examined on the 58th day, the mean whole-blood concentration had increased by 67 per cent. and the plasma concentration by 25 per cent. of the respective resting values (minimal concentrations).

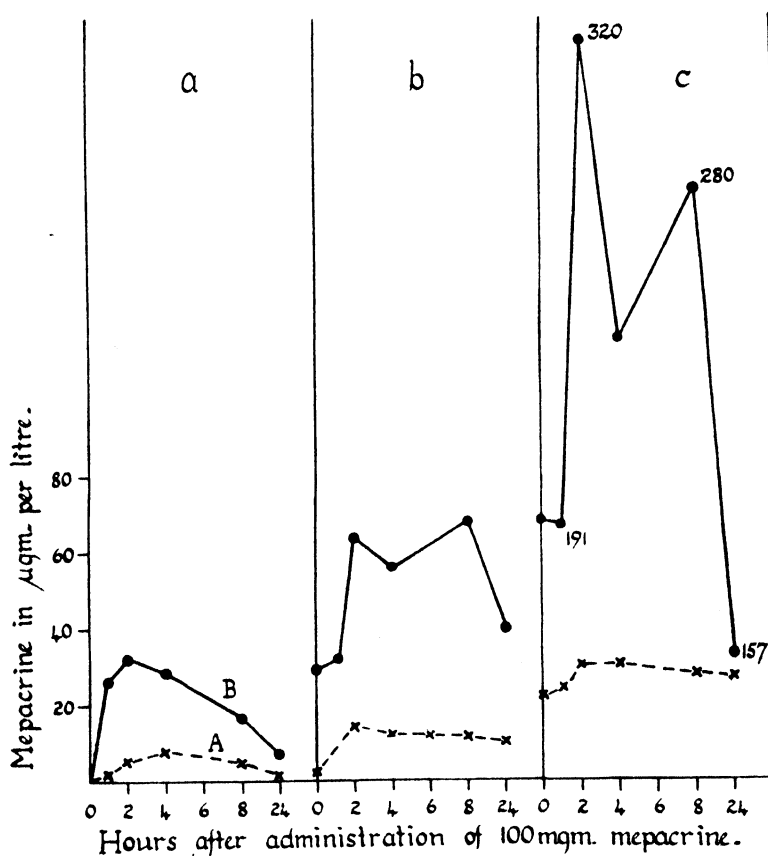


FIG. 1. Plasma and whole-blood post-absorption curves.

a.—First day on 100 mgm. mepacrine hydrochloride daily (nine subjects).

b.—Seventh day on 100 mgm. mepacrine hydrochloride daily (nine subjects).

c.—Fifty-eighth day on 100 mgm. mepacrine hydrochloride daily (four subjects).

A.—Plasma concentrations (dashed line).

B.—Whole-blood concentrations (continuous line).

SUMMARY

The post-absorption curve of plasma concentration following oral administration of 100 mgm. mepacrine reached its peak in 2–4 hours after dosage. The degree of rise at the peak period appears to be independent of the plasma concentration obtaining at the time of administration.

II. WHOLE-BLOOD AND PLASMA CONCENTRATIONS REACHED DURING THE STANDARD SUPPRESSIVE COURSE OF MEPACRINE (100 MGM. MEPACRINE HYDROCHLORIDE DAILY)

Minimal whole-blood and plasma concentrations* reached on a dosage of 100 mgm. mepacrine hydrochloride daily were measured at intervals in two groups of undergraduate volunteers.

The first group consisted of 16 men who took the drug regularly for periods varying from three to 10 months, and from whom blood samples were taken at frequent intervals. The second group consisted of 25 women from whom blood samples were taken at six-weekly intervals. All the women volunteers took the drug for six months, and nine of them continued the dosage régime for a total of 10 months or more (Army Malaria Research Unit, 1946b).

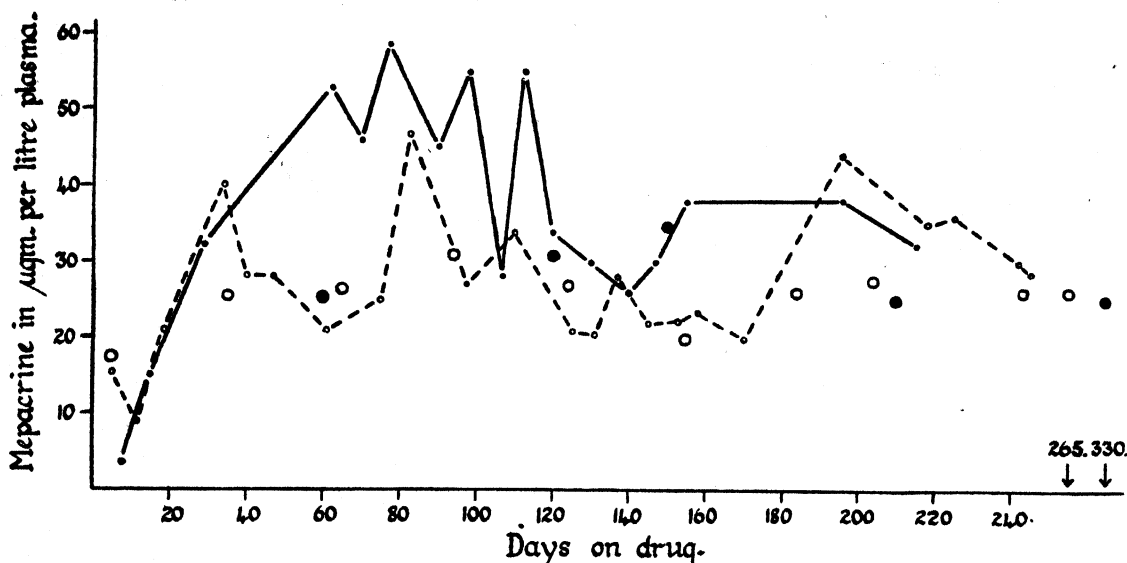


FIG. 2. Plasma concentrations reached on suppressive régime of 100 mgm. mepacrine hydrochloride daily.
 ○ Mean concentrations in 16 men.
 ● Mean concentrations in 23 women (last point mean of nine only).
 ○ Volunteer 260.
 ● Volunteer 288.

The mean (arithmetic) plasma concentrations reached during the dosage régime in both groups are shown in fig. 2. The average mean concentration found after the 8th week of dosage was 26.0 μ gm. per litre in the men and 28.0 μ gm. per litre in the women. These average means compare well with the mean of 25.3 μ gm. per litre observed by Brown and Rennie (1945) in 29 individuals in Italy who had been taking 100 mgm. mepacrine for 5-6 months. Fairley (1945) does not give the average arithmetic mean concentration reached in his 35 subjects, but the mean_G in his experiments was of the same order, 22.5 μ gm. per litre.

* Minimal concentrations are defined as those concentrations reached on the dosage régime in question 24 hours after taking 100 mgm. (i.e., immediately prior to a dose).

The average mean plasma concentration was reached in our men volunteers between the 4th and 5th weeks after the start of dosage. The curve of mean plasma concentrations then tended to rise slightly above the average mean value, returning to it by the 6th month. This rise following the attainment of the average mean concentration was often clearly seen in individual cases (fig. 2). A similar rise has also been noted by Fairley (1945). In Fairley's subjects the return to the average mean concentrations was more rapid than in our volunteers.

There was considerable variation about the mean in individual subjects. Some reached the mean concentration rapidly, others oscillated about it for many weeks before reaching equilibrium. Once equilibrium was established, however, the plasma concentration tended to remain fairly steady and the equilibrium concentration was maintained, no consistent rise or fall being seen in any of our subjects over the period of observation.

After dosage was stopped, the fall-off in plasma concentration occurred at about the same rate as the initial build-up, mepacrine usually being absent from the plasma by the 6th week after stopping the drug.

Our results correspond closely with those of Fairley (1945) in Australia, of Brown and Rennie (1945) in Italy, and of Shannon (1943) and the Fort Knox workers (1943) in America, in that the equilibrium concentration reached on suppressive dosage, once established, was maintained over the period of the dosage. There is no evidence to indicate that the plasma mepacrine concentration falls off appreciably if the subject is taking his dose of drug regularly. It can be assumed from this that during the suppressive régime the plasma concentration reached, after the initial period of build-up, will be sufficient continuously for the suppression of malaria (Fairley regards a mean_G concentration of 20 µgm. per litre as adequate for this purpose).

We have stated elsewhere reasons for considering that whole-blood mepacrine concentrations do not give so reliable an indication of the physiological availability of the drug as plasma concentrations (Army Malaria Research Unit, 1946*a*). In these experiments, however, the whole-blood concentrations were measured in the group of men volunteers at the same time as the plasma concentrations. As was anticipated, the individual variations were extreme and the (arithmetic) mean values at given times varied widely. Thus, after three months the mean for all subjects was 180 µgm. per litre; after six months, 120 µgm. per litre; and after nine months, 160 µgm. per litre (five subjects only). The average mean concentration reached after the 6th week following the start of dosage was 150 µgm. per litre. A rise above this mean value and a fall below it, followed by restoration and maintenance of the level, corresponded closely with similar variations in the plasma concentration curve. This undulation in the curve probably accounts for the 'progressive fall from the peak level to one of about 100 µg. per litre at 6 months after dosage began' described by Reid (1945). The cause of this variation in mean concentration is obscure. In the light of what has been said concerning the plasma concentrations obtaining during the suppressive dosage régime, we do not consider that these variations in whole-blood concentrations are of any significance in relation to suppression of malaria.

SUMMARY

The plasma and whole-blood concentrations reached on an oral dosage of 100 mgm. mepacrine daily were measured at intervals for periods of 3-10 months in 16 men and 25 women undergraduates.

The average mean plasma concentrations reached in the men and women were 26.0 and 28.0 μ gm. per litre respectively. Equilibrium values, once established, were well maintained.

The average mean whole-blood mepacrine concentration reached in the men was 150 μ gm. per litre.

III. THE CONCENTRATIONS OF MEPACRINE IN THE BLOOD AND PLASMA OF PATIENTS AND VOLUNTEERS DURING A STANDARD COURSE OF THERAPY

This report records observations on the mepacrine concentrations in whole blood, plasma and urine in patients suffering from proved benign tertian malaria and in healthy volunteers, all of whom received the following dosage régime :

First two days : 200 mgm. six-hourly.
Next 10 days : 100 mgm. three times daily.
Total : 4.6 gm. mepacrine in 12 days.

EXPERIMENTAL

The subjects of this study formed three groups :

1. Four healthy volunteers, three of whom had never received mepacrine previously, and one of whom had not received it for six months.
2. Two healthy volunteers who had received 100 mgm. mepacrine daily for the two months preceding the start of the experimental therapeutic course.
3. Sixty-seven soldiers suffering from proved benign tertian malaria, treated in general hospitals in Oxford, Colchester and Halton. Most of this group, which included Italian prisoners-of-war, had previously been on some form of suppressive antimalarial therapy, which, except in one case, had ceased at least a month before the present attack of malaria.

Blood samples were withdrawn from subjects immediately before the administration of the morning dose at 09.00 hours, the dosage schedule being so arranged that on the first two days the subject received his last dose of 200 mgm. (completing the 800 mgm. for the day) at 03.00 hours, and on the next 10 days the last dose of 100 mgm. (completing the 300 mgm. for the day) at 21.00 hours. Blood was oxalated and doubly centrifuged as soon as possible. Samples of plasma from patients in hospitals outside Oxford were sent by post and extracted on the day following the withdrawal of the blood. In the six healthy volunteers blood and plasma concentrations were followed closely for the first 12 hours of the dosage régime.

Twenty-four-hour urine samples were also collected.

Mepacrine estimations were made by a modification of the method of Masen (1943).

RESULTS

1. *Volunteers.* The blood and plasma concentrations observed in these subjects are given in Tables I, II and III and in fig. 3.
2. *Patients.* The results are considered in two groups. Group A includes observations on 28 patients in a general hospital in Oxford, on whom daily measurements of

TABLE I

Mean blood and plasma concentrations of mepacrine in healthy volunteers for first two days of régime.
Four volunteers who had not previously taken the drug

Time	Day 1						Day 2	
	09.15 hrs.	09.30 hrs.	10.00 hrs.	12.00 hrs.	15.00 hrs.	19.00 hrs.	09.00 hrs.	19.00 hrs.
Blood ($\mu\text{gm./l.}$)	3	6	34	49	85	151	249	236
Plasma ($\mu\text{gm./l.}$)	9	9	25	27	23	20	30	57

TABLE II

Plasma concentrations of mepacrine ($\mu\text{gm./l.}$) in healthy volunteers

Four normal			Previously saturated	
End of day	No.	Mean	Volunteer 250	Volunteer 252
Immediately before 1st tablet			58	31
Day 1	4	27	135	52
2	4	75	114	79
3	4	68	128	80
4	2	43	215	83
5	4	62	124	86
6	4	62	—	—
7	4	53	204	83
8	4	53	143	66
9	4	67	149	93
10	2	63	104	97
11	—	—	235	97
12	2	49	—	—

TABLE III

Whole-blood concentrations of mepacrine ($\mu\text{gm./l.}$) in healthy volunteers

Four normal			Previously saturated	
End of day	No.	Mean	Volunteer 250	Volunteer 252
Immediately before 1st tablet			498	249
Day 1	4	246	870	456
2	4	396	1,320	720
3	4	337	—	532
4	4	311	1,280	587
5	4	326	177	600
6	4	294	—	—
7	4	333	1,380	570
8	4	319	614	547
9	3	355	1,132	740
10	4	382	1,082	636
11	—	—	1,280	665
12	—	—	—	—

blood, plasma and urine mepacrine concentrations were made. Group B includes observations made on the 1st, 2nd and 12th days of the therapy course in all patients, including those in group A. Blood and plasma concentrations for groups A and B are shown in Tables IV and V and in fig. 4. The urinary output is detailed in Table VI.

TABLE IV
Infected patients

End of day	No.	Whole-blood concentrations ($\mu\text{gm./l.}$)			
		Mean	\pm SD.	Maximum	Minimum
GROUP A					
1	27	157	150	795	35
2	28	257	136	470	35
3	26	405	—	840	131
4	28	428	—	1,270	97
5	25	446	—	1,180	166
6	27	445	—	930	115
7	24	516	—	1,090	134
8	23	477	—	770	103
9	26	531	—	1,500	110
10	23	534	—	1,010	190
11	20	581	—	1,765	170
12	18	551	242	1,020	290
GROUP B					
1	50	136	113	795	35
2	51	261	120	535	22
12	40	598	282	1,380	179

TABLE V
Infected patients

End of day	No.	Plasma concentrations (μgm./l.)			
		Mean	± SD.	Maximum	Minimum
GROUP A					
1	26	30.5	19.1	84	2
2	28	87.5	45.9	197	21
3	25	97.0	—	207	5
4	23	89.8	—	206	28
5	26	80.9	—	188	34
6	27	65.9	—	165	12
7	27	56.9	—	97	25
8	23	61.7	—	137	19
9	24	61.0	—	94	12
10	23	63.6	—	156	17
11	24	60.9	—	250	24
12	17	67.5	22.8	132	45
GROUP B					
1	61	31.9	21.2	121	2
2	67	83.4	36.2	197	9
12	53	65.2	20.7	132	20

TABLE VI

Total amount of drug excreted (mgm.) in urine of infected patients

Day	No.	Mean	Maximum	Minimum
GROUP A				
1	4	0.49	1.1	0.2
2	20	6.91	43.0	0.6
3	19	9.62	18.6	1.6
4	22	9.91	17.8	0.7
5	22	8.76	20.0	2.8
6	23	12.53	45.1	1.7
7	24	7.22	17.2	1.8
8	20	9.54	21.5	2.9
9	21	9.69	40.5	1.3
10	18	6.85	13.1	2.5
11	13	13.33	31.5	0.9
12	17	9.91	17.1	4.0
GROUP B				
1	22	2.99	11.6	0.2
2	45	9.14	43.0	0.3
12	44	9.90	20.1	2.8

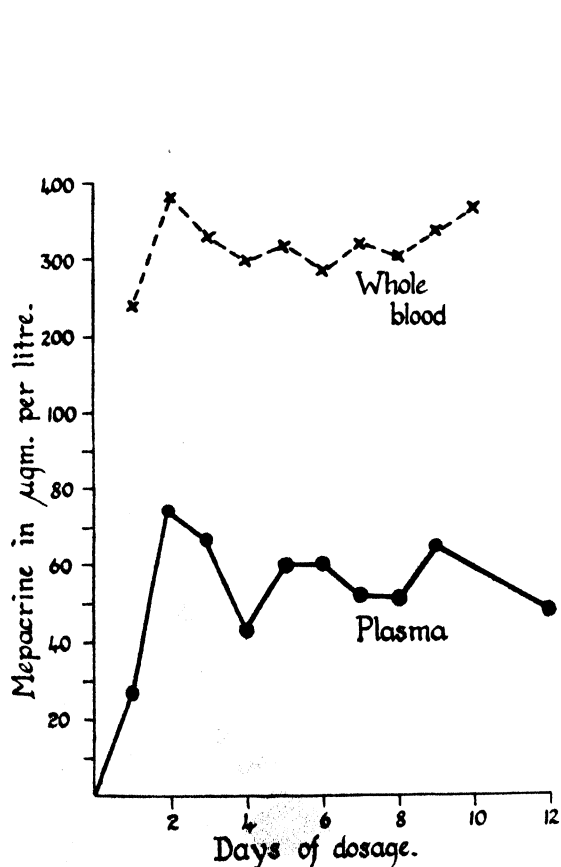


FIG. 3. Mean whole-blood and plasma concentrations reached in four healthy volunteers taking therapeutic régime of mepacrine hydrochloride (800 mgm. for two days, followed by 300 mgm. for 10 days).

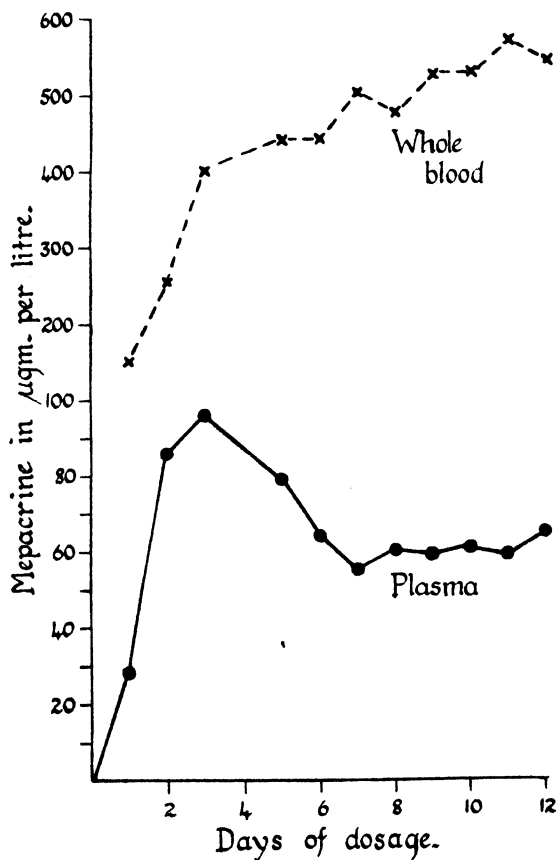


FIG. 4. Mean whole-blood and plasma concentrations reached in 28 patients suffering from benign tertian malaria taking a therapeutic régime of mepacrine hydrochloride (800 mgm. for two days followed by 300 mgm. for 10 days).

DISCUSSION

The results show a wide variation in individual responses to the same dosage—a feature that has been observed by other workers (Shannon, 1943). This variation is well seen in Tables IV and V, while examples of high and low mean mepacrine concentrations in patients are shown in fig. 5. It is interesting to note that the patient Jones, who exhibited the low levels, stated that he had been receiving 0.2 gm. mepacrine twice a week until the onset of his attack, whereas the patient Asaro, with the high levels, denied previous suppressive therapy.

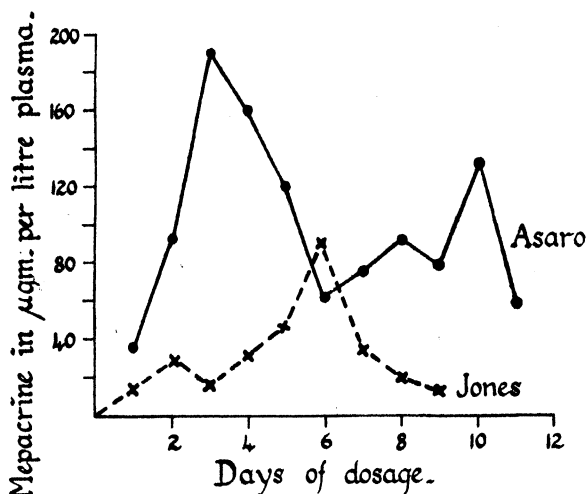


FIG. 5. Examples of individual extreme high and low plasma concentrations reached after therapeutic dosage of mepacrine.

The mean daily plasma mepacrine concentrations of patients (group A) are graphed in fig. 4. It will be seen that the plasma levels reach their highest points on the 2nd, 3rd and 4th days and then subside to a lower steady level. A similar peak is observed in the volunteers' curve (fig. 3). It was found that for the patients in group B the level on day 2 was significantly ($P = <0.01$) higher than that on day 12. This peak, the existence of which can be taken as real, is evidently determined by the interaction of various factors, which must include the absorption of the drug from the gut, the change in daily intake of the drug from 800 mgm. to 300 mgm., and the slow translation of the free drug in the plasma into the tissues. There may be a later redistribution of the drug, involving transfers back from the tissues to the plasma.

It is evident that the establishment of an equilibrium between the drug in the plasma and that in the blood-cells is slow, since it has been found that during the last five days of the course the whole-blood concentration continues to increase although the mean plasma concentration is virtually constant (fig. 4).

If the mean values for the groups are considered, it is clear that the plasma concentration attained on an actively suppressive régime (i.e., about 25 µgm. per litre) is reached rapidly and maintained throughout the peak period. On the 12th day the mean concentration reached was 65.2 ± 20.7 µgm. per litre, a value well above this suppressive concentration.

The average daily excretion of the drug in the urine of the patients undergoing the therapy course was about 10 mgm. (see Table VI), but the daily output in individual patients is subject to considerable fluctuations, probably resulting from changes in ammonia excretion (Army Malaria Research Unit, 1945).

SUMMARY

1. The mean plasma mepacrine concentration finally attained in a group of 67 patients suffering from benign tertian malaria who had undergone a therapeutic course of 4.6 gm. mepacrine given over 12 days was 65.2 μ gm. per litre \pm 20.7, with a range of 130–20 μ gm. per litre.
2. The mepacrine concentration in the plasma on the 2nd day of the group of infected patients was significantly higher than that attained on the 12th day.
3. Changes in plasma concentrations are not immediately followed by similar changes in the whole-blood concentration.
4. There was considerable individual variation in response to the dosage.

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THE EFFECT OF VARIOUS SUBSTANCES, INCLUDING FOOD, ON THE ABSORPTION OF ORALLY ADMINISTERED MEPACRINE HYDROCHLORIDE

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The experiments described here were carried out in order to determine the effect of food on the rate and completeness of the absorption of mepacrine after oral administration. The object of the first part of the work was simply to decide experimentally whether the drug should be given on a full or on an empty stomach. As will be seen, it was found that there was no significant difference between the plasma levels reached when the drug was given regularly in between meals or with food. The presence of large amounts of cellulose in the diet did not affect this result. The effects on the absorption of mepacrine of certain commonly used therapeutic agents, i.e., kaolin, magnesium trisilicate and McLean's powder, were also studied, and here again it was shown that the administration of these substances did not influence the plasma concentration reached.

The results of these experiments have been interpreted in terms of the absorption of the drug from the gut, on the assumption that the so-called 'post-absorption' curves of plasma concentrations do, in fact, at any one time represent the balance reached between the absorption, distribution and excretion of the drug after oral administration. If this assumption be accepted, our results indicate in general that the absorption of mepacrine from the gut is not easily disturbed, so that the plasma concentrations reached on a given dosage régime in any individual case are likely to be fairly constant. It is more difficult to argue from this point to the suppression of malaria, but, if such suppression is related in some way to plasma mepacrine concentration, as is generally agreed, it is reasonable to suppose that the suppressive power of the dosage régime under question is also unaffected by the various routines examined in this series of experiments.

I. THE EFFECT OF FOOD ON THE ABSORPTION OF MEPACRINE

EXPERIMENTAL

Three groups of four Army volunteers were used. Groups I and II were given 100 mgm. mepacrine daily for a week on an empty stomach and on a full stomach respectively. For the next three weeks no mepacrine was given, after which the dosage was resumed for 14 days with the groups reversed, so that those who had been taking the drug on an empty stomach now took it with food, and vice versa. The third group (group III) was given 100 mgm. mepacrine daily on an empty stomach for 14 days.

When the drug was given on an *empty stomach* it was given at 10.45 hours, i.e., three hours after breakfast and two hours before lunch. When it was given on a *full stomach* it was given at 08.00 hours, during breakfast. A draught of water was taken after each

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tablet. The administration of the tablets was personally supervised in each case by a sergeant, an entry being made in each man's record-book after each dose.

The subjects of this experiment were Army privates in good health. History, physical examination, repeated blood counts, liver and renal function tests and gastro-intestinal examination by means of test-meals, barium meals and gastroscopy revealed no abnormality in any case which might have influenced the absorption of mepacrine. No symptoms were reported during the investigation, which was begun in the middle of January and completed by the middle of March, 1944. During this time the men performed light duties.

MEPACRINE ESTIMATIONS

Daily venipunctures were performed immediately before the administration of each dose of mepacrine. In addition, on the 1st, 7th and 14th days, the whole-blood and plasma post-absorption curves were traced, samples being taken 1, 2, 4 and 8 hours after the giving of the tablets. Blood was also taken for mepacrine estimation on alternate days during the first week after cessation of the drug. Mepacrine was estimated in oxalated blood and plasma by the modified method of Masen (1943).

Twenty-four-hour specimens of urine, for estimation of the total excretion of mepacrine, were collected during the first half of the experiment in groups I and II and throughout the experiment in group III.

RESULTS

(a) Mean Mepacrine Concentration in Plasma and Whole Blood

A summary of results is given in Table I and fig. 1. The mean concentration of mepacrine in plasma and whole blood was not significantly lower on the 8th and 15th days when the tablets were given on a full rather than an empty stomach.

On the 15th day the mean plasma concentration of mepacrine in the empty-stomach group was 16.5 μ gm. per litre, and that in the full-stomach group 10.2 μ gm. per litre. This difference was not significant ($P = 0.4$). The figures for concentrations in whole blood on the 15th day showed the same trend, but the difference was less marked.

TABLE I
Mean mepacrine concentrations in plasma and whole blood

Subjects	8th day Concentrations in μ gm./l.		15th day Concentrations in μ gm./l.	
	Plasma	Whole blood	Plasma	Whole blood
Full stomach	9.3 \pm 2.6	49.3 \pm 7.8	10.2 \pm 3.8	63.7 \pm 3.2
Empty "	8.5 \pm 1.5	45.4 \pm 5.6	16.5 \pm 6.1	74.6 \pm 13.3
Calculated values (Fort Knox, 1943)	10.5	—	15.7	—

(b) Post-Absorption Curves in Plasma and Whole Blood

On the first day of dosage the concentration of mepacrine in both plasma and whole blood was lower during the post-absorptive period in the full-stomach group than in the empty-stomach group. The difference between the mean plasma concentrations in the two groups was significant ($P = <0.05$).

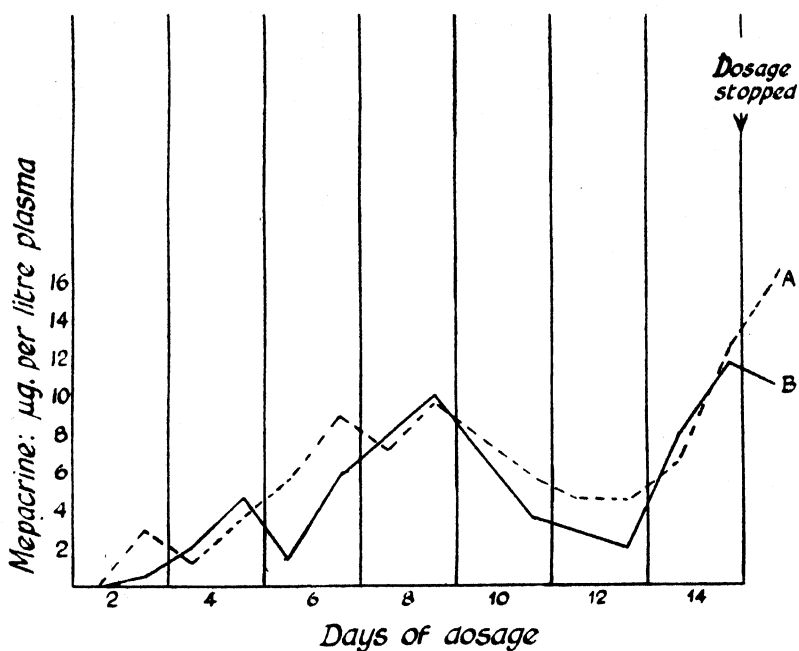


FIG. 1. Mean minimal plasma mepacrine concentrations.

A.—Drug taken on empty stomach (dashed line). B.—Drug taken on full stomach (continuous line).

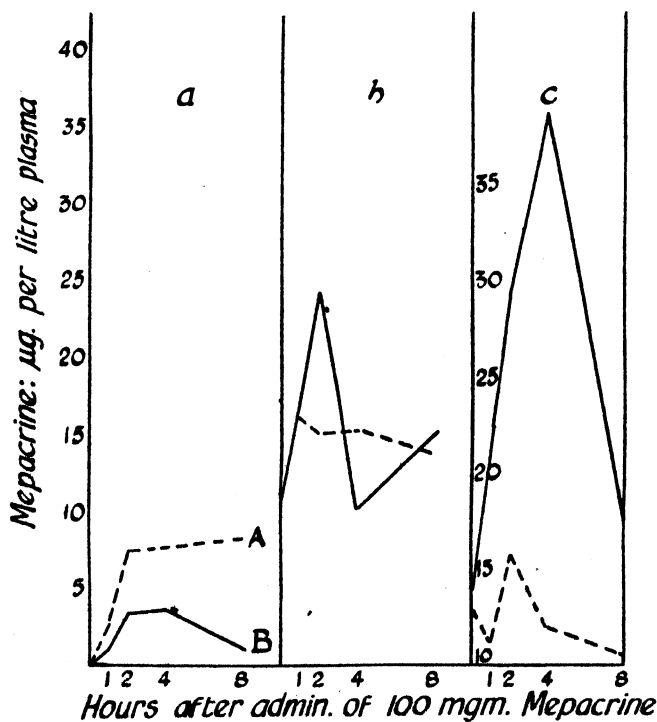


FIG. 2. Plasma post-absorption curves.

a.—First day on 100 mgm. daily. b.—Seventh day on 100 mgm. daily. c.—Fourteenth day on 100 mgm. daily.
A.—Drug taken on empty stomach (dashed line). B.—Drug taken on full stomach (continuous line).

On the 7th day the plasma post-absorption curves were almost identical in the two groups. On the 14th day the post-absorption curve was lower than it had been previously in the empty-stomach group; in the full-stomach group it was higher. The difference between the two groups was significant ($P = <0.05$) (fig. 2).

The figures for the concentration of mepacrine in whole blood showed the same tendency as the figures for plasma concentrations, but the difference between the two groups of subjects was less marked on the first day, and data on the 14th day were incomplete.

(c) *Output of Mepacrine in the Urine*

The total output of mepacrine in the urine during each 24 hours was significantly less in the full-stomach group than in the empty-stomach group. After cessation of the dosage the difference between the output in the two groups diminished as time went on.

DISCUSSION

Assuming that the suppressive value of mepacrine is related to its concentration in the plasma, the efficiency of any method of administration of the drug may be measured by the mean plasma concentration reached in a group of subjects on a given dosage régime. Using this criterion, our results show that giving the drug on a full stomach is as efficient a method as giving it on an empty stomach, the latter method being the one way in which *a priori* it might be expected that absorption would be more rapid and possibly more complete. Apparently, therefore, absorption is not impaired when the drug is mixed in the stomach with the ordinary home-scale Army diet. There is thus no objection, on the grounds of absorption, to administering the drug in this way.

There is evidence, however, in the post-absorption curves that there is an initial difference in the speed of absorption of mepacrine from a full and an empty stomach, even though the degree of absorption appears to be finally the same in the two groups of subjects, as indicated by the ultimate plasma concentrations reached. Thus, the post-absorption curve after the first tablet is significantly higher in the empty-stomach group and significantly lower in the same group after the 14th tablet. The change from the first to the 14th dose is also significant within each group.

The full interpretation of the post-absorption curve is obscure, but certain points are clear. The mepacrine level in plasma during the first few hours after oral administration of mepacrine must depend, amongst other things, on the speed of absorption from the gut and the speed of disappearance from the plasma. The excretion through the kidney on a dose of 100 mgm. mepacrine daily is so small that its influence on the post-absorption curve must be negligible (Army Malaria Research Unit, 1945). Thus the changes we have observed in the post-absorption curves must be explained partly by the differences in speed of absorption from the gut or by the differences in speed of loss from the plasma to the tissues. If it is assumed that the changes which we have seen have been due to differences in the speed of absorption, our results indicate that on the first day absorption was slower on a full stomach, but that, during a period of 14 days on a régime of 100 mgm. mepacrine daily, some change took place which led to quicker absorption from the full stomach. We have no information regarding the mechanism by which this change in rate of absorption could take place.

The differences in the excretion of mepacrine in the urine in the two groups of subjects may have been associated with the differences in post-absorption plasma levels seen in these groups at the beginning of the dosage régimes, since, other things being equal, the urinary output is closely related to plasma concentration (Army Malaria Research Unit, 1944). There may, however, have been differences in ammonia production in the two experimental groups, and hence in mepacrine output; but, unfortunately, these experiments were carried out before the relation between ammonia excretion and mepacrine output was discovered, and there are no records of ammonia output or the reaction of the urine passed during the experiments.

The mean plasma mepacrine concentrations reached in both the full- and the empty-stomach groups correspond fairly closely to the theoretical values for the dosage calculated by the Fort Knox method (1943). In the experience of these workers, the equilibrium concentration reached on any dosage may be derived from the equation:

Plasma level, in $\mu\text{gm. per litre} = 30 \times \text{dose per week (in grams)}$.

By the end of the first week approximately 50 per cent. of this level has been attained, and approximately 75 per cent. by the end of the second week (see Table I).

II. THE EFFECT OF FOOD RICH IN CELLULOSE ON THE ABSORPTION OF MEPACRINE

It was shown in the previous section that the diet of the soldier in England did not interfere with the absorption of mepacrine from the gut. The high incidence of malaria in the troops invading Sicily in 1943, however, gave rise to the suggestion that the apparent failure of mepacrine as a suppressive drug in this campaign might have been related to the high cellulose content of the Sicilian diet. It was known that cellulose adsorbs mepacrine readily *in vitro*, and it was thus possible that the drug became fixed by adsorption to the cellulose in fruit and vegetables (which were consumed in large quantities by the troops) and so was rendered less readily available for absorption. The experiments recorded here put this suggestion to the test, with negative results.

EXPERIMENTAL

Four healthy Army volunteers were given a diet rich in cellulose for a preliminary period of three days and throughout a three-week course of 100 mgm. mepacrine daily. The diet contained large amounts of fresh and canned vegetables and was supplemented by 10 oranges a day. The dose of mepacrine was taken during breakfast. The minimal mepacrine concentrations were determined on the 7th, 14th and 21st days, and plasma concentrations during the post-absorptive period were measured on the 1st, 7th and 14th days.

RESULTS

The minimal plasma concentrations of mepacrine during and at the end of the three-week period of the high-cellulose diet did not differ significantly from those seen in the same four men during two previous experiments, in which similar dosages were taken on an empty and on a full stomach respectively (see Section I of this paper).

The plasma concentrations found during the post-absorptive period on the cellulose diet were higher than those in the experiments in which the drug was taken on an empty stomach, and higher than those seen on the 1st and 7th days when the drug was taken on a full stomach, but lower than those seen on the 14th day on a full stomach (fig. 3).

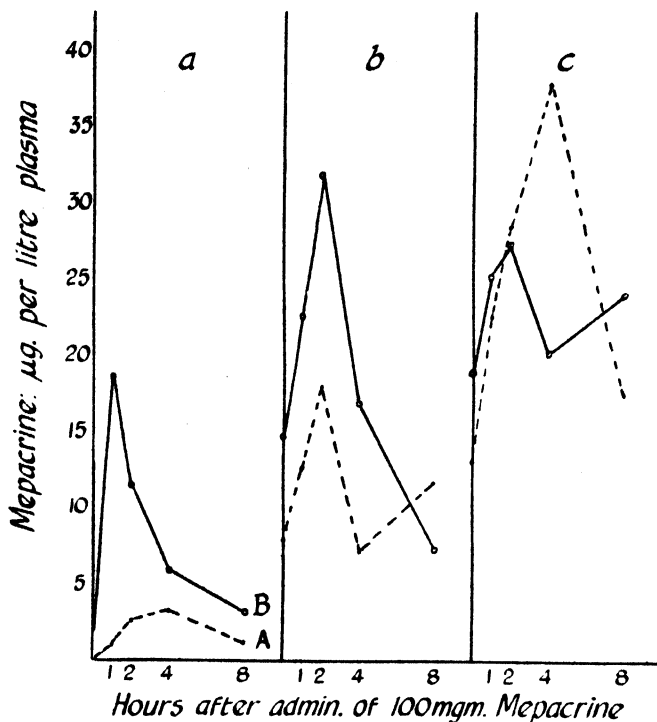


FIG. 3. Plasma post-absorption curves.

a.—First day on 100 mgm. daily. b.—Seventh day on 100 mgm. daily. c.—Fourteenth day on 100 mgm. daily.
A.—Mepacrine taken with ordinary diet. B.—Mepacrine taken with high-cellulose diet.

These results have been analysed by calculating the area of each post-absorption curve. The area of the curve was calculated from the sum of the mepacrine plasma concentrations at zero time (*a*), 1 hour (*b*), 2 hours (*c*), 4 hours (*d*), and 8 hours (*e*) after taking 100 mgm. mepacrine, using the formula

$$\text{Area} = \frac{a}{2} + b + \frac{3c}{2} + 3d + 2e \quad \dots \quad (1)$$

The results, shown in Table II, are therefore given in terms of micrograms mepacrine absorbed per litre of plasma per hour. As can be seen from the table, the post-absorptive mepacrine concentration in the plasma was higher while the men were taking a cellulose-rich diet than it was during the other experiments, with the exception of the concentration seen on the 14th day of the 'full-stomach' experiment.

The curious observation that cellulose, which adsorbs mepacrine so vigorously *in vitro*, appears to aid the absorption of the drug *in vivo* is not understood. Such increased absorption is not seen with ordinary food at the beginning of a course of mepacrine, although, at the end of a fortnight on 100 mgm. mepacrine daily, ordinary food does lead to the appearance of higher plasma concentrations than are seen when the drug is taken on an empty stomach (see Section I of this paper).

Whatever the explanation of the enhancement of mepacrine absorption in the presence of cellulose, it is a point of practical importance that the absorption of mepacrine is not at any rate *hindered* by the presence of cellulose in the diet.

TABLE II

Mean area of post-absorption curves under different dietary conditions. Mepacrine 0.1 gm. daily

Method of administration	Mean area of post-absorption curve, $\mu\text{gm./l./hour}$		
	1st day	7th day	14th day
On an empty stomach	42.2 ± 11.2 (10 subjects)	92.1 ± 25.5 (9 subjects)	99.8 ± 15.6 (8 subjects)
On a full stomach (ordinary diet)	17.5 ± 5.2 (8 subjects)	91.8 ± 12.2 (8 subjects)	214.0 ± 18.0 (4 subjects)
On a full stomach (high-cellulose diet)	64.1 (4 subjects)	142.3 (4 subjects)	185.7 (4 subjects)

III. THE EFFECT OF KAOLIN, MAGNESIUM TRISILICATE AND McLEAN'S POWDER ON THE ABSORPTION OF MEPACRINE

Because mepacrine is adsorbed readily by substances such as silica and alumina, the suggestion has been made that some of the powders which were administered liberally in malaria endemic areas to men with gastro-intestinal disturbance might adsorb mepacrine, thus making it unavailable to the epithelium and impairing its absorption from the gut. If this were so, the prescription of such powders would be contra-indicated in cases where it is important to maintain chemical protection against malaria. In the experiments discussed here, three 'stomach-powders' were administered to groups of volunteers receiving 100 mgm. mepacrine daily, and the absorption of mepacrine in terms of plasma concentrations was compared with that seen in controls taking mepacrine only.

Preliminary *in vitro* experiments showed that each of the three powders selected for this trial (kaolin, magnesium trisilicate and McLean's powder) adsorbed large quantities of mepacrine from solutions, the concentrations of which corresponded roughly to what might be expected to be found in the stomach and small intestine after oral administration of 100 mgm. For example, if 1 gm. of kaolin is added to 20 c.cm. of 1 per cent. solution of mepacrine and the suspension shaken gently for half an hour, the concentration of the supernatant fluid after centrifuging is reduced to about 0.25 per cent. Magnesium trisilicate removes even more of the drug from solution. The results of *in vitro* experiments are shown in Table III.

TABLE III

Adsorption of mepacrine from 1 per cent. solution by various alkaline powders

	Mepacrine adsorbed	Mepacrine remaining in solution
Magnesium trisilicate ...	80 per cent.	20 per cent.
" oxide ...	99.9 "	0.1 "
" carbonate ...	87 "	13 "
Bismuth " ...	92 "	8 "
" salicylate ...	92 "	8 "
Calcium carbonate ...	99 "	1 "
Kaolin	77 "	23 "
McLean's powder ...	55 "	45 "

It was recognized that, before conclusions could be drawn from these experiments which would be applicable to absorption *in vivo*, it would be necessary to study the adsorption by kaolin, etc., at different degrees of acidity and in different concentrations, and to determine how difficult was the elution of the drug once adsorption had occurred. The experiments did, however, give support to the suggestion that these powders might interfere with absorption, and it was decided to proceed with *in vivo* trials rather than to carry on with studies *in vitro*, the results of which would in the end still have to be submitted to trial *in vivo*.

EXPERIMENTAL

Kaolin, magnesium trisilicate and McLean's powder were selected for trial because they are commonly used in the treatment of dyspepsia. Two groups of four volunteers and one group of three volunteers—one group on each powder—were given one drachm of powder after each meal for a period of two weeks, during which they received mepacrine 100 mgm. daily, and for the two days immediately preceding these two weeks. Mepacrine was taken after breakfast at the same time as the powder. The minimal plasma concentration of mepacrine was determined on the 7th and the 14th days. On the 1st, 7th and 14th days the post-absorptive concentrations of mepacrine in the plasma were determined 1, 2, 4 and 8 hours after taking the tablet.

The controls were eight volunteers who in previous experiments had received 100 mgm. mepacrine daily on a full stomach (see Section I of this paper). The experimental group taking kaolin constituted four of the controls.

RESULTS

The minimal plasma concentrations of mepacrine in the subjects taking kaolin and McLean's powder were higher than those in the controls at the end of the first week. The plasma concentrations in the subjects taking magnesium trisilicate were low at the end of the first week, but at the end of the second week the concentrations were approximately the same as those in the controls.

The plasma concentrations of mepacrine 1, 2, 4 and 8 hours after administration of the tablet and the 'stomach-powder' were higher on the first day in all groups of subjects than in the controls. On the 7th day the groups on kaolin and McLean's powder had higher plasma mepacrine concentrations than the controls, and the group on magnesium trisilicate had concentrations about equal to those seen in the controls. On the 14th day the post-absorption curves in the groups on magnesium trisilicate and McLean's powder were lower than, and in the group on kaolin about equal to, those seen in the controls. The areas of the post-absorption curves in each case (calculated from the formula given in Section II of this paper) are set out in Table IV, together with the mean of the areas of the post-absorption curves in the controls. Figs. 4, 5 and 6 show the post-absorption curves in the different groups; the control-groups have been included for comparison.

DISCUSSION

The point of practical importance in the results of this experiment is the observation that the contemporaneous administration of any of these three 'stomach-powders' does not lead to a minimal plasma concentration of mepacrine which is lower than that seen in controls. There is therefore no contra-indication to their use in persons who are receiving suppressive mepacrine.

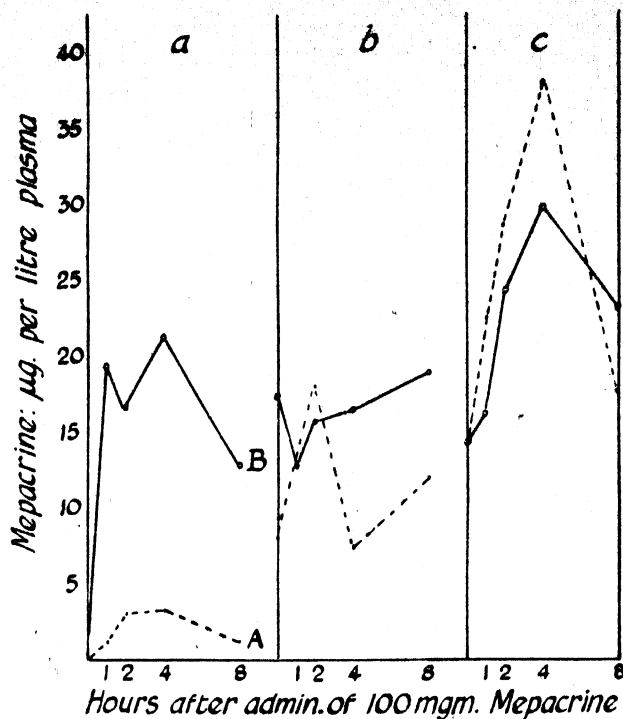


FIG. 4. Plasma post-absorption curves.

a.—First day on 100 mgm. daily. *b*.—Seventh day on 100 mgm. daily. *c*.—Fourteenth day on 100 mgm. daily.
A.—Mepacrine taken with ordinary diet. B.—Mepacrine taken with ordinary diet plus kaolin.

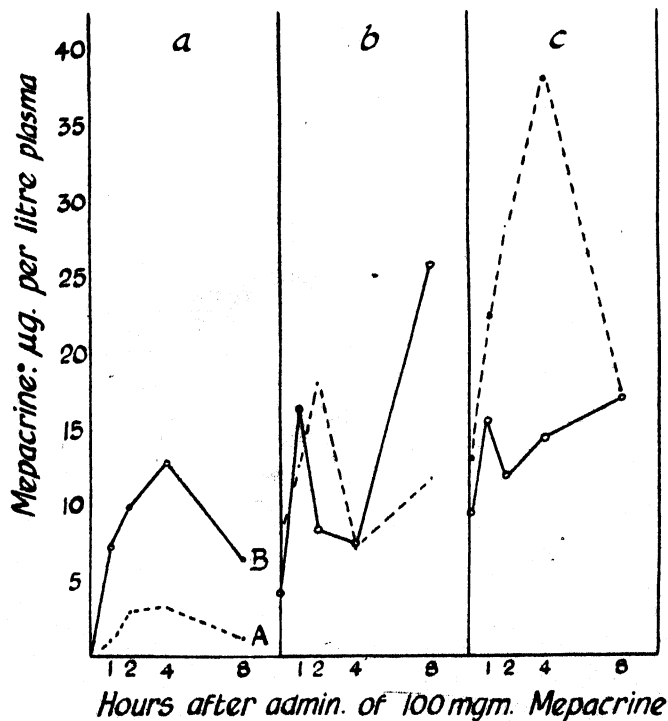


FIG. 5. Plasma post-absorption curves.

a.—First day on 100 mgm. daily. *b*.—Seventh day on 100 mgm. daily. *c*.—Fourteenth day on 100 mgm. daily.
A.—Mepacrine taken with ordinary diet. B.—Mepacrine taken with ordinary diet plus magnesium trisilicate.

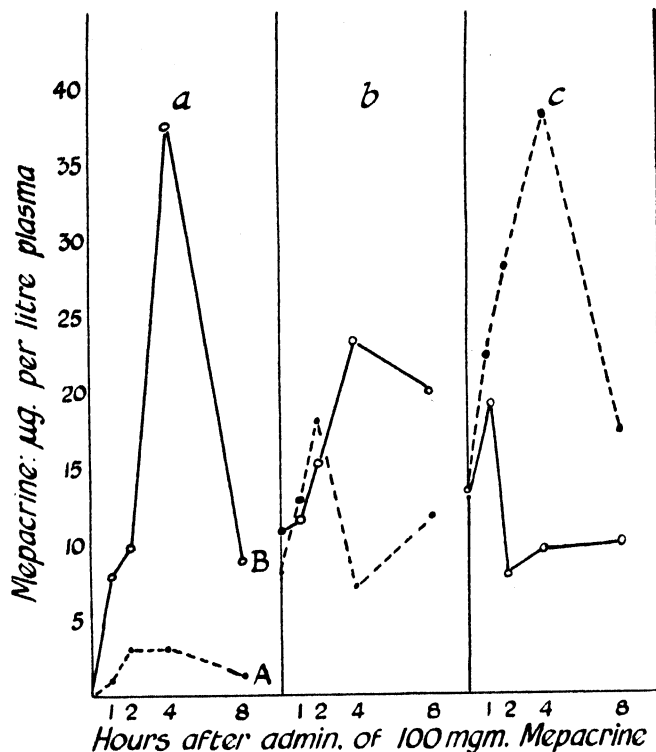


FIG. 6. Plasma post-absorption curves.

a.—First day on 100 mgm. daily. *b.*—Seventh day on 100 mgm. daily. *c.*—Fourteenth day on 100 mgm. daily.
A.—Mepacrine taken with ordinary diet. *B.*—Mepacrine taken with ordinary diet plus McLean's powder.

TABLE IV

Areas of the post-absorption curves during treatment with 'stomach-powders' and in controls.
 Mepacrine 0.1 gm. daily

Method of administration		Area of post-absorption curve, $\mu\text{gm.}/\text{l.}/\text{hour}$		
		1st day	7th day	14th day
Controls (means), full stomach ...		17.5 (8 cases)	91.8 (8 cases)	214.0 (4 cases)
With kaolin after breakfast	Case 1	97	151	299
	" 2	127	204	164
	" 3	133	1,100	137
	" 4	173	—	165
	Mean	132	152	191
With magnesium trisilicate after breakfast	Case 1	73	68	115
	" 2	74	151	90
	" 3	64	90	—
	Mean	70	103	102
With McLean's powder after breakfast	Case 1	235	190	84
	" 2	72	128	94
	" 3	133	113	74
	" 4	161	160	70
	Mean	150	148	81

The differences which have been observed in the post-absorption curves should be considered with the results of the experiments described in Sections I and II of this paper. In none of these experiments was the final plasma concentration of mepacrine reached in a given time significantly raised or lowered, indicating that absorption of the drug was probably complete in all cases. An examination of the post-absorption curves suggests, however, that the *speed* of absorption may be influenced by the contents of the gastro-intestinal tract. Absorption after the first dose of mepacrine is slower if it is taken with food, but, in the presence of excess of cellulose or of kaolin, magnesium trisilicate and McLean's powder, the initial rate of absorption is faster. The effect of ordinary food is not unexpected, but why substances which adsorb mepacrine *in vitro* should aid absorption *in vivo* is difficult to understand.

SUMMARY

1. The plasma mepacrine concentrations reached after a fortnight on a daily dose of 100 mgm. are not affected by the presence of food in the stomach. Food has, however, an effect on the rate of absorption of the drug from the gut.

2. The presence of large amounts of cellulose in the food does not affect the total absorption of mepacrine.

3. Kaolin, magnesium trisilicate and McLean's powder, which all adsorb mepacrine *in vitro*, do not affect the total absorption of the drug *in vivo*. These substances appear, however, to increase the rate of absorption of mepacrine on the first day of administration.

4. Food, cellulose, kaolin, magnesium trisilicate and McLean's alkali powder can all be given to subjects on a suppressive régime of 100 mgm. mepacrine hydrochloride daily without fear of seriously affecting the ultimate minimal plasma levels reached.

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THE ABSORPTION AND EXCRETION OF PALUDRINE IN THE HUMAN SUBJECT

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Some aspects of the pharmacology of Paludrine in relation to its use as an antimalarial have been referred to in previous communications (Adams, Maegraith *et al.*, 1945; Maegraith, Adams *et al.*, 1945; Maegraith, Adams *et al.*, 1946). This paper deals with the results of experiments designed to study in more detail in human subjects the absorption and excretion of Paludrine and its distribution in the elements of the blood. The work has been carried out in both normal subjects and patients suffering from benign tertian or malignant tertian malaria. In the earlier experiments the drug was administered in the form of the acetate, but, following satisfactory clinical results obtained with the more easily prepared hydrochloride, the latter salt has now been adopted for general use, and in all the experiments referred to below the hydrochloride has been used.

In our experiments on the absorption and excretion of Paludrine we have dealt almost exclusively with plasma concentrations. This has been done because we consider that the plasma concentration of a drug which, like Paludrine, is distributed in a definite proportion between plasma protein and plasma water gives a better indication of its concentration in tissue fluid than does any other easily available measurement (Shannon, 1943). For the ordinary run of experiments it is impracticable to measure directly the concentration of a drug in solution in the plasma water.

In considering the relation of the blood and tissue concentration of Paludrine to its antiplasmodial action, we have assumed that the concentration in the tissue fluid is the best estimate of the availability of the drug in the body to the various stages of the developing malaria plasmodium. If this assumption is correct, the plasma concentration, as representing the nearest estimate of concentration of the drug in the tissue fluid, should be closely related to the degree of antimalarial activity of the drug.

The relation between plasma concentration and antiplasmodial activity in antimalarial drugs has been most thoroughly studied in the case of mepacrine. Fairley (1945) found that suppression of malaria was complete in a group of subjects with a mean_G plasma concentration of 22.9 μ gm. per litre, with a very wide individual variation of plasma concentration ranging between 7.0 and 90.0 μ gm. per litre. After cessation of dosage, relapses of benign tertian malaria occurred in Fairley's cases; measurement of the mepacrine plasma concentration on the day following the appearance of a proved relapse

showed a group mean concentration of 3.4 $\mu\text{gm.}$ per litre. In a series of relapses of benign tertian malaria occurring in British troops in England in 1943-44 following mepacrine therapy, the plasma mepacrine concentrations were found to range from 2 to 21 $\mu\text{gm.}$ per litre (Army Malaria Research Unit, 1944). The plasma concentrations reached during effective therapeutic doses of mepacrine are uniformly higher than these (Army Malaria Research Unit, 1946c). These and similar findings indicate that plasma mepacrine concentrations can be broadly related, over a wide range of individual values, to the suppressive and therapeutic activity of the drug, although no well-defined critical concentration can be demonstrated above which plasmodia are controlled and below which development can take place.

In the case of Paludrine we have not such extensive evidence, but there appears little doubt that its suppressive and therapeutic activity is related in some degree to its concentration in the plasma. As will be seen below, we have been handicapped in our experiments by the fact that, at the lower therapeutically active dosages, the plasma concentrations obtaining are at the limits of the technique of estimation.

TECHNIQUE

Paludrine hydrochloride was administered orally in the form of tablets followed by a draught of water, the administration being supervised by a senior member of the nursing staff. A solution of the hydrochloride supplied by Imperial Chemical Industries Limited was used for intravenous injection.

At intervals after the dose had been given 20 ml. blood samples were withdrawn and oxalated. Plasma was separated immediately by centrifugation and collected with due protection against contamination by blood-cells. Urine samples were collected under toluene and stored in the cold. If analysis could not be carried out at once, the plasma samples were stored in the refrigerator until required. Under these conditions there was no apparent change in the amount of Paludrine recoverable from plasma or urine samples.

Samples of tissue obtained at post-mortem were extracted as described by Spinks and Tottey (1946a) for animal tissue.

In the experiments in which the concentration of Paludrine in faeces was determined, the material was stored in the refrigerator in glass jars and dispersed in water before analysis. Analyses were performed on samples of freshly stirred emulsions. In experiments in which only one dose of Paludrine was given, faeces were collected over each 24 hours. In the longer experiments, in which the faecal excretion was measured over a full therapeutic 14-day régime, faeces were collected and stored in glass jars over periods of three days until the cessation of dosage, and thereafter daily. It was shown, by repeated experiments, that Paludrine is not destroyed by contact with faeces after 1-4 days, either in the dark, in the light, at room-temperature or when incubated at 37° C.

The Paludrine concentration of all samples was determined by the method of Spinks and Tottey (1946a).

PLASMA CONCENTRATION CURVES FOLLOWING THE ADMINISTRATION OF SINGLE DOSES OF PALUDRINE ORALLY

After administration of the drug, blood samples (20 ml.) were withdrawn into oxalate at intervals of 1, 2, 4, 8, 12, 24 and 48 hours. The plasma was centrifuged off and its Paludrine content was measured.

The doses investigated in this way were 500, 400, 300, 200, 100 and 50 mgm. Paludrine. Concentrations were measured for each dose in three different patients or volunteers. Representative examples of the concentration curves obtained with each dose are given in fig. 1.

It will be seen from the figure that for all doses from 500 to 100 mgm. the maximum concentration observed was reached four hours after the administration of the drug, the height of this maximum depending largely upon the size of the dose.

It is probable that a similar maximum was also attained at about four hours after doses lower than 100 mgm. (see fig. 1), but the concentrations reached after such small doses are near the limits of the analysis technique, and measurement of them is therefore

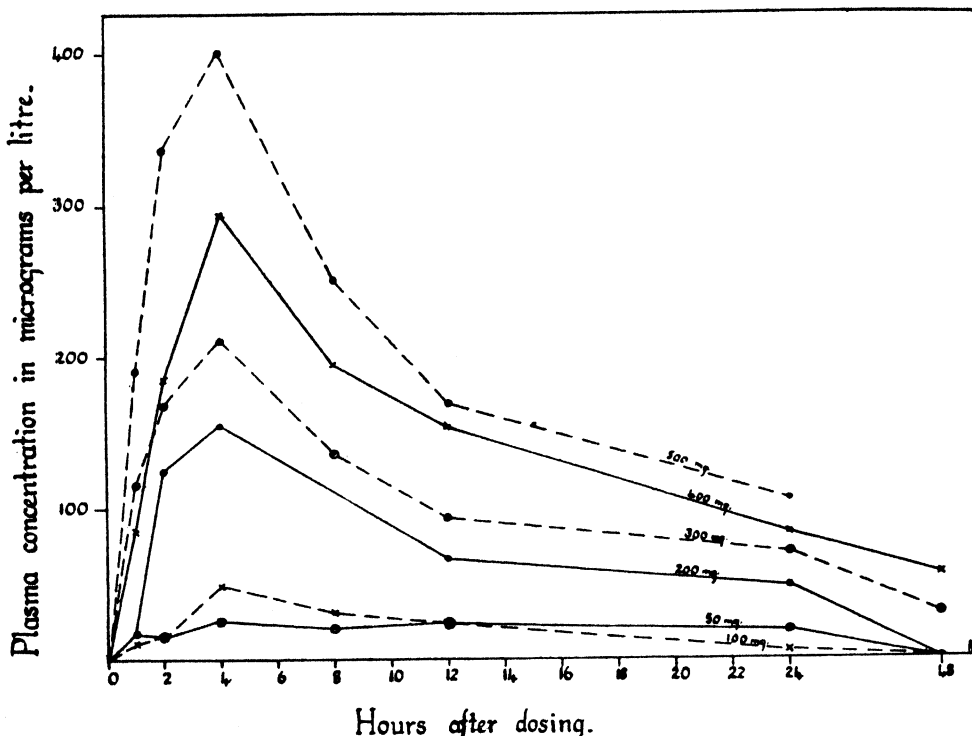


FIG. 1. Plasma concentration curves following single oral doses of Paludrine. The size of the dose is marked on the respective curves.

not very accurate except when large samples of plasma are used. At each dose-level there was some individual variation between the three subjects in the maximum attained and the rate of disappearance of the drug from the plasma, but such variation was not pronounced. It will be noted that the plasma concentration after 24 hours is also related to the size of the dose. As has been pointed out elsewhere, this persistence of the drug in the plasma following the higher single doses is probably of therapeutic importance (Maegraith *et al.*, 1946).

Although detailed experiments were not carried out at doses below 50 mgm., the clinical activity of amounts as small as 5 mgm. made it important to ascertain the plasma concentrations obtaining at these low doses. Estimations of plasma concentrations in these

cases were made on plasma from single samples of 60 ml. blood withdrawn at the expected peak period, four hours after dosage. The results are set out in Table I. The concentration of 6 $\mu\text{gm.}$ per litre attained after 5 mgm. of Paludrine seems very low for therapeutic activity, but, if in these particular patients a therapeutically active plasma concentration be admitted, it must be at or below the concentration measured. If the therapeutically active concentration does in fact lie in this region, the effect of a single dose of the order of 500–100 mgm. must be exerted on the parasite for periods of many hours (Maegraith *et al.*, 1946).

In a few experiments whole-blood concentrations of Paludrine were estimated at the same time as the plasma concentrations. In any given subject the ratio between the two sets of concentrations was nearly constant, varying between 2:1 and 4:1 in different subjects.

It will be noted that for corresponding dosages of Paludrine the plasma concentrations reached are higher than for most other antimalarials—for example, for mepacrine (Shannon, 1943) and for 3349 (Spinks and Tottey, 1945b).

TABLE I
Plasma concentrations following single oral doses of 5 and 10 mgm. Paludrine

Experiment no.	Plasma concentration 4 hours after oral dose of	
	10 mgm.	5 mgm.
1	22 $\mu\text{gm.}/\text{l.}$	6 $\mu\text{gm.}/\text{l.}$
2	17 $\mu\text{gm.}/\text{l.}$	6 $\mu\text{gm.}/\text{l.}$

PLASMA CONCENTRATIONS ATTAINED DURING A THERAPEUTIC COURSE OF PALUDRINE

The therapeutic courses used in these experiments were those referred to in our previous communications, viz., 500 mgm. or 50 mgm. given twice daily at 12-hourly intervals for 14 days. In all cases plasma estimations were made as nearly as possible 12 hours after the preceding dose of drug had been administered. The concentration reached at that time is taken to represent the minimal concentration in the plasma during the 12-hour period.

Fig. 2 illustrates the minimal plasma concentrations reached during the full course of 14 days in two subjects, one taking 500 mgm. and the other 50 mgm. twice daily. The plasma concentrations observed in the seven days following cessation of dosage are also shown in both cases.

Examination of the figure shows that an irregular 'plateau concentration' is reached at about the third day. This plateau concentration has been found to average between 300 and 500 $\mu\text{gm.}$ per litre during a course of 500 mgm. twice daily, and between 50 and 150 $\mu\text{gm.}$ per litre on a course of 50 mgm. twice daily, although considerable individual variation occurs outside these limits. After cessation of dosage the fall-off in plasma concentration is rapid, the concentration falling below the limits of the analysis method within 3–7 days. The disappearance of the drug from the plasma is more rapid on the lower dosage.

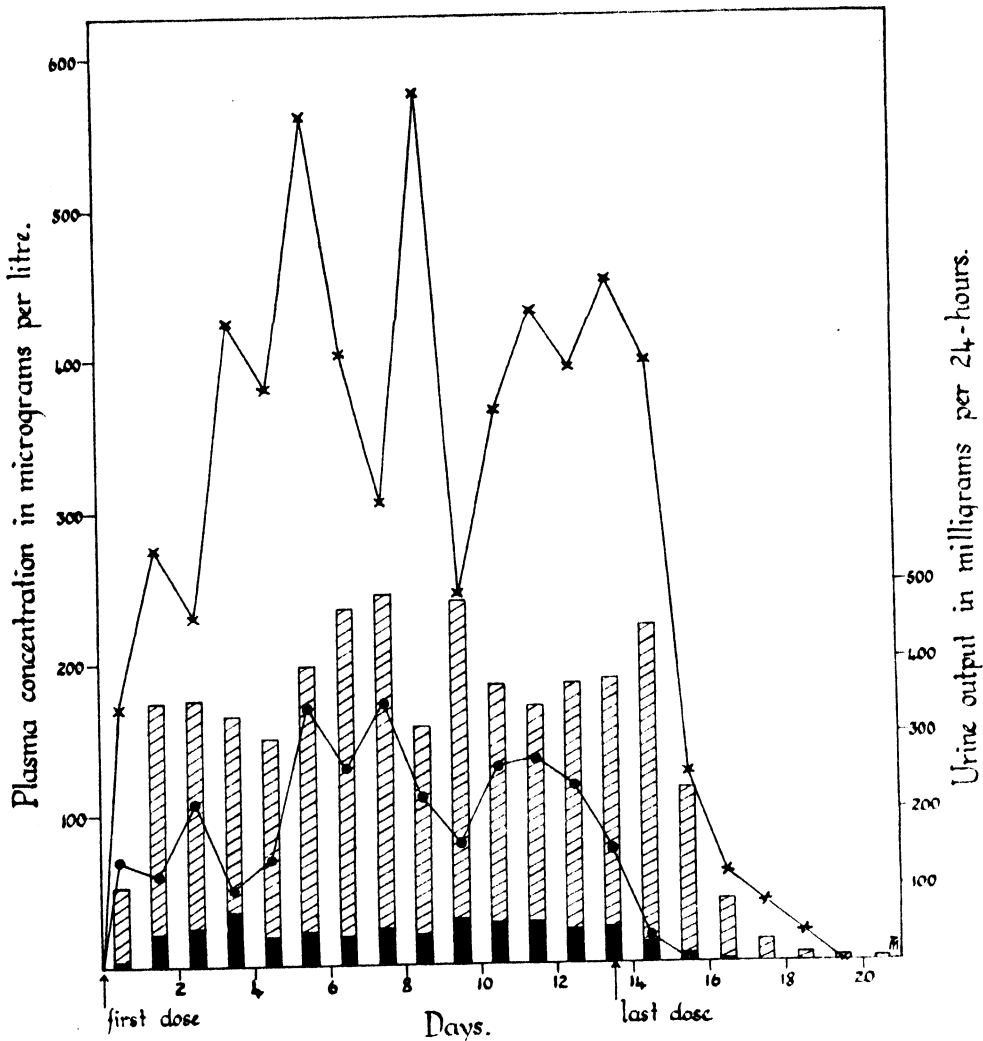


FIG. 2. Plasma 'resting' concentration curves and daily urinary excretion of Paludrine during a 14-day course of 500 mgm. and 50 mgm. b.d.

× — × Plasma concentrations on 500 mgm. Paludrine b.d.
 ● — ● " " " 50 mgm. " "
 ■ Urinary output " 50 mgm. " "
 // " " 500 mgm. " "

The plateau concentration may be taken as representing the balance for the dosage concerned between the intake of the drug, its fixation in the blood-cells and tissues, and its degradation and excretion. The plateau is reached more rapidly with Paludrine than with mepacrine, so that, bearing in mind the extremely low concentration of Paludrine found in clinically effective low dosage, it is probable that a 'build-up' or 'loading dose' of Paludrine is unnecessary in the treatment of benign tertian malaria. The plateau concentration attained with Paludrine is higher than that reached with equivalent doses of

mepacrine. Thus, on 100 mgm. mepacrine daily the concentration reached after 4-6 weeks averages about 25 μ gm. per litre (Shannon *et al.*, 1944; Fairley, 1945; Maegraith and Havard, 1945), compared with 50-150 μ gm. per litre reached in the course of a few days after the equivalent dose of Paludrine. Experiments have been carried out to ascertain whether there is any adaptation of the body to the drug detectable in the form of changes in the absorption curve during a course of therapy. Plasma concentrations were measured 1, 2, 4, 8 and 12 hours after administration of a tablet to patients who had been taking 500 mgm. or 100 mgm. Paludrine twice daily for a period sufficiently long to allow the

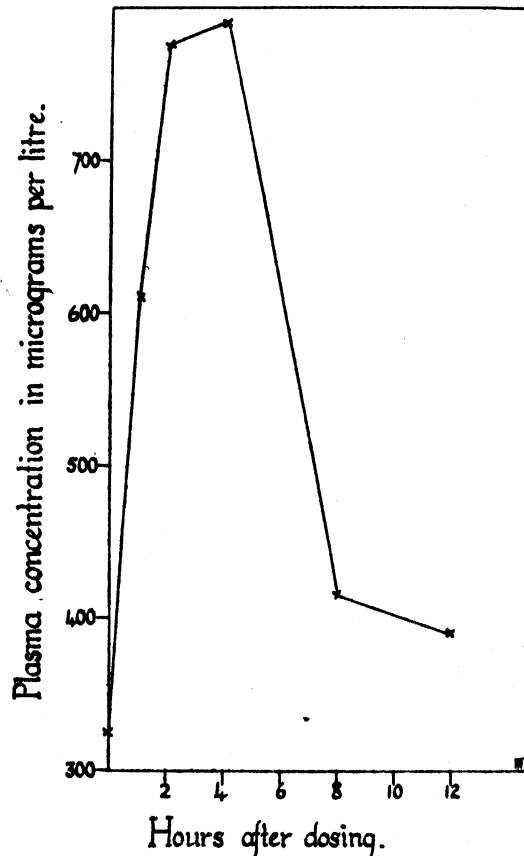


FIG. 3. Plasma concentration curve following the 20th dose of Paludrine during a course of 500 mgm. b.d.

development of a plateau concentration. Fig. 3 illustrates an absorption curve obtained following a dose of 500 mgm. Paludrine administered to a patient taking 500 mgm. twice daily. It will be seen that the absorption curve is indistinguishable from that seen in fig. 1 following a single dose of 500 mgm. It is thus apparent that the existence of a plateau concentration in the plasma does not affect the absorption and subsequent plasma concentration reached following further dosage. This indicates that the tissues can in no sense be 'saturated' with the drug even on the relatively high dosage of 500 mgm. twice daily, and that the degradation and excretion of the drug apparently continue normally under such conditions.

The disappearance of Paludrine from the plasma after cessation of dosage is rapid (fig. 2). As will be seen later, the drug is often detectable in the urine for a day or so later than in the blood, but it is evident that for all practical purposes it has disappeared from the body within a week of the cessation of dosage (Adams *et al.*, 1945). This rapid disappearance contrasts with the fate of mepacrine, which can be detected in both blood and urine for a considerable time after cessation of treatment. According to workers at the Armored Medical Research Laboratory in America (1943), mepacrine concentration in the plasma falls off after stoppage of treatment at a rate of roughly 50 per cent. every seven days. The disappearance of Paludrine from the blood-stream is very much faster than this, owing partly to its more complete excretion in the urine and possibly partly to its being less firmly fixed or more rapidly degraded in the tissues than mepacrine.

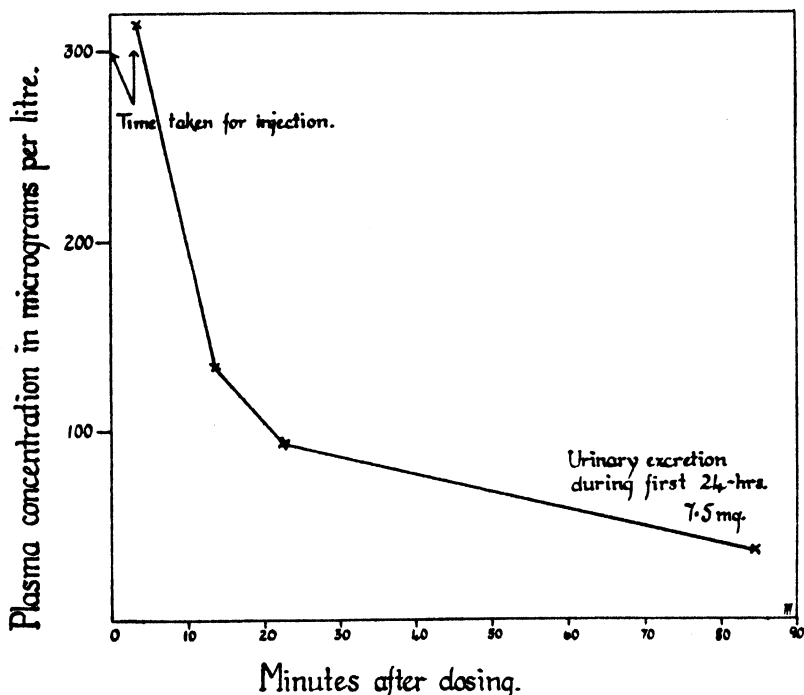


FIG. 4. Plasma concentration curve and urinary excretion following an intravenous injection of 20 mgm. Paludrine.

PLASMA CONCENTRATIONS FOLLOWING SINGLE INTRAVENOUS INJECTIONS OF PALUDRINE

Paludrine was injected intravenously in four cases of neurosyphilis undergoing courses of therapeutic trophozoite-induced benign tertian malaria. The first dose given was 5 mgm., administered in 10 ml. physiological saline over a period of five minutes. This dose was calculated to give an initial plasma concentration of the same order as those attained in the maximum oral dosage used. No toxic effects were noted as a result of this injection, and subsequently intravenous doses of 10 mgm., 20 mgm. and 50 mgm. were administered, similarly without toxic effects.

As soon as possible after dosage, blood was withdrawn from the opposite arm, and thereafter further samples were withdrawn at frequent intervals. The plasma Paludrine concentration was measured in all samples, the whole-blood concentration in some. The result of an experiment in which 20 mgm. were given intravenously is shown in fig. 4. The figure shows a very rapid fall of plasma concentration following injection. This rapid disappearance of Paludrine from the plasma is probably partly indicative of a very rapid fixation in the tissues, such as is known to occur with other antimalarials (Shannon *et al.*, 1944; Army Malaria Research Unit, 1946a; Chen and Geiling, 1944; Spinks and Tottey, 1946b) and has been demonstrated in the case of Paludrine in the rat and the mouse (Spinks, *in the press*). The fall of plasma concentration is, however, also related to the rapid removal of the drug through the kidneys—for example, in an experiment in which 50 mgm. Paludrine was injected intravenously, 6.65 mgm. was excreted in the urine during the first 90 minutes.

In one experiment whole-blood Paludrine concentrations were measured in addition to the plasma concentrations. It was anticipated that the whole-blood and plasma concentration curves might diverge with increasing time following the injection, if the process of fixation in the tissues and white cells was not very rapid. This was found not to be the case; the ratio of whole-blood concentration to plasma concentration remained fairly constant throughout the experiment at between 2 : 1 and 3 : 1. This result is in agreement with the conclusions already drawn from the rapid disappearance of the injected Paludrine from the plasma. The process of fixation in the blood-cells and tissues appears to be a very rapid one.

THE DISTRIBUTION OF PALUDRINE IN HUMAN TISSUES AND BLOOD

Examination of Paludrine concentration in certain tissues was made possible as the result of the death of two patients who were being treated with the drug. One case of neurosyphilis died 36 hours after intravenous injection of 5 mgm. Paludrine; death was due to intercurrent streptococcal septicaemia. The other death occurred from cerebral malaria after the patient had been given 900 mgm. Paludrine in the course of 24 hours. The distribution of Paludrine in the organs of these two cases closely resembled that observed in animals (Spinks, *in the press*). The drug was most concentrated in the kidney and next in the liver. In this respect Paludrine differs from mepacrine and 3349, which are most concentrated in the liver (Army Malaria Research Unit, 1946a; Spinks and Tottey, 1946b). The relatively high concentration of Paludrine in the kidney is possibly associated with the active renal excretion of the drug.

It has already been stated that the concentration of Paludrine in whole blood varies from two to four times that in the plasma in any given subject. This increase in concentration in whole blood in relation to that in plasma is due to fixation in the blood-cells, and an attempt was therefore made to determine more precisely the distribution of the drug between the cellular elements. The importance of such distribution has been shown in the case of mepacrine, where the fixation of the drug in the white cells is so great that the concentration in the blood is much more closely related to that in the white cells than to that in either the plasma or the red cells (Army Malaria Research Unit, 1946b).

The distribution of Paludrine in human blood has been studied in patients receiving the full 14-day course of 500 mgm. twice daily. Blood samples (60 ml.) were drawn into oxalate 14 hours after a dose of 500 mgm. had been administered, in order that the distribution might have reached equilibrium. Whole blood was analysed for Paludrine in

triplicate, and the remainder of the blood was centrifuged in tubes made with long capillary waists, as described elsewhere (Army Malaria Research Unit, 1946*b*), the volume placed in each tube being so calculated from the volume of the bulb and the haematocrit values that the leucocytes collected in the capillary waist relatively uncontaminated by red cells. Cell-free plasma and packed red cells were analysed in triplicate, and suspensions of leucocytes, obtained from a known volume of blood, in duplicate. Knowing the haematocrit values and the red- and white-cell counts of the original whole blood, it was possible to express the Paludrine content of the three elements of the blood in terms of percentage of whole-blood content. (This method has been applied elsewhere to the investigation of the distribution of mepacrine in blood; see Army Malaria Research Unit, 1946*b*.) The result of two experiments are set out in Table II, together with those of a comparable experiment in which mepacrine was used. In case 1 the leucocyte count was 6,400 per c.mm., so that the content of Paludrine per white cell was 120 times that in an erythrocyte. In case 2 the leucocyte count was 6,450, the content per white cell being 54 times that in an erythrocyte.

TABLE II
The distribution of Paludrine and mepacrine in blood

	Paludrine				Mepacrine	
	Experiment 1		Experiment 2		$\mu\text{gm./l. of blood}$	Percentage of blood content
	$\mu\text{gm./l. of blood}$	Percentage of blood content	$\mu\text{gm./l. of blood}$	Percentage of blood content		
Erythrocytes	831	73	820	79.4	28	13.5
Plasma ...	154	13.6	147	14.2	21	10
Leucocytes	152	13.4	66	6.4	159	76.5

In these experiments it was not possible to compare the concentrations in polymorphonuclear cells and lymphocytes, but later, through the kindness of Professor Henry Cohen, a case of lymphatic leukaemia was made available to us. The patient was not treated with Paludrine, but the drug was added in therapeutic concentration to a 100 ml. sample of oxalated blood and the mixture was maintained at 37° C. for two hours before analysis. The state in which the drug exists in the body is unknown, but in this case it was added as a solution of the base. The distribution of Paludrine was determined as in the previous experiments, and the results are shown in Table III.

TABLE III
Distribution of Paludrine in leukaemic blood

	Paludrine as	
	$\mu\text{gm./l. of blood}$	Percentage of whole-blood content
Erythrocytes ...	200	20.5
Plasma ...	244	25.1
Leucocytes ...	529	54.4

In this case the white-cell count was 70,000 (over 80 per cent. lymphocytes) and the erythrocyte count 2.8 million per c.mm. Thus, each white cell contained 73 times the amount of drug held by an erythrocyte. This figure is in good agreement with that obtained from the previous experiments, so that it may be assumed that the drug is probably equally distributed between polymorphonuclears and lymphocytes. The low-percentage drug content of the red-cell fraction in this case must be attributed to the low red-cell count. In order to compare the Paludrine concentration in unit volumes of red and white cells, allowance should be made for the considerably larger volume of the white cells.

Although we have not been able to repeat this experiment, the results obtained on this isolated case show that the whole-blood concentration of Paludrine must be dependent to some extent on the number of leucocytes present, although not so much as in the case of mepacrine, where the individual white-cell concentration is of the order of 3,000 times that in the red cell (Army Malaria Research Unit, 1946b).

THE DISTRIBUTION OF PALUDRINE BETWEEN THE PROTEIN AND WATER OF PLASMA

Reference has already been made to the probable physiological and therapeutic significance of the concentration of Paludrine in plasma water. Direct separation of the fixed protein-bound and free drug by dialysis is impracticable in the case of antimalarial drugs, as they are mostly adsorbed on semi-permeable membranes. Measurement of these fractions of Paludrine solution in plasma was therefore made by the indirect method employed by Shannon *et al.* (1944) in the case of mepacrine.

Equal numbers of washed red cells were suspended in equal volumes of both plasma and Tyrode solution in physiological proportions, and equal volumes of a solution of the drug in the form of the base was added to each set of red-cell suspensions. The mixtures were left to equilibrate at 23° C. for two hours and were then centrifuged; the Paludrine concentrations of both supernatant fluid and packed red cells were determined.

Assuming that the red cells were in equilibrium with the same concentration of the drug in simple aqueous solution in both the Tyrode and the plasma, the difference in drug content in these two media after separation of the red cells can be considered to give a measure of the protein-binding of the drug taking place in the plasma.

The results of our experiments show that at 23° C. in normal plasma about 70–80 per cent. of the Paludrine in solution is bound, presumably to the protein. It will be noted that this is the same degree of binding as is found with mepacrine (Shannon *et al.*, 1944). The result was the same in the one experiment so far carried out at 37° C.

THE EXCRETION OF PALUDRINE IN THE URINE

It was pointed out in a previous communication (Adams, Maegraith *et al.*, 1945) that, on a dosage of 500 mgm. or 50 mgm. Paludrine daily for 14 days, between 30 and 50 per cent. of the drug is excreted in the urine. Urinary excretion has been further studied in most of the subjects referred to in this paper and in over 100 patients treated with Paludrine for benign or malignant tertian malaria.

The excretion of Paludrine following single oral doses of 500–10 mgm. is shown in Table IV. For doses of 500–50 mgm. the figures in the table were obtained at the same time as the plasma concentration curves for these doses, which are illustrated in fig. 1.

It will be seen that after single doses urinary excretion of the drug amounted to between 40 and 60 per cent. of the administered dose. The amount of drug in the urine fell to less than 1 per cent. of the administered dose in from five to three days after dosage, the period decreasing with the dose.

After full therapeutic dosage régimes of 500 mgm. to 50 mgm. twice daily for 14 days, the drug disappears from the urine in 6-9 days and 4-6 days respectively. The excretion after repeated dosage thus continues for very little longer than after single doses of the same magnitude. We interpret this finding as indicating that the drug stored in the tissues during continuous dosage is not very firmly fixed and is probably degraded rapidly.

In comparison with mepacrine and 3349, Paludrine is more rapidly and more completely eliminated by the kidneys. Experiments by Spinks and Tottey (1945a) showed that urinary excretion of 3349 in the human amounted to only 4 per cent. of the intake, and had not become constant by the fifth day after dosage of 500 mgm. twice daily. Following cessation of this dosage, the drug was still detectable in the urine after 23 days.

TABLE IV
Urinary excretion of Paludrine in mgm./24 hours following single oral doses of the drug

Time	Dose administered in mgm.							
	500	400	300	200	100	50	25	10
1st 24 hours ...	190.5	198.0	129.0	72.0	40.3	11.9	3.2	2.8
2nd " ...	54.2	20.6	28.2	26.3	7.0	4.8	1.9	0.4
3rd " ...	10.7	13.7	7.7	8.2	6.2	1.4	0.5	0.3
4th " ...	9.4	6.5	1.3	4.3	4.7	1.0	0	0
5th " ...	4.3	1.1	0.86	2.1	1.0	0.5	0	0
Total ...	269.1	239.9	167.06	112.9	59.2	19.6	5.6	3.5
Percentage of dose	53.8	60.0	55.7	56.5	59.2	39.3	22.6	35.0

Mepacrine behaves similarly to 3349 (Henry and Grindley, 1945) and has been detected in urine up to 70 days after therapeutic dosing (Farinaud *et al.*, 1937). On the other hand, the excretion of quinine in the urine is similar to that of Paludrine, 25-66 per cent. of the intake being eliminated in the urine after oral dosage, the drug disappearing from the urine in two days after a single dose and in about a week after repeated dosing (Ramsden *et al.*, 1918).

It must be emphasized here that the hydrolysis followed by the diazo reaction used in the present method of analysis of urine during these experiments is given, not only by Paludrine itself, but by any metabolic product containing an aromatic amine residue. Our figures therefore represent the amount of Paludrine itself plus possible degradation products, calculated as Paludrine.

Attempts have been made to correlate the urinary concentrations and output of Paludrine with plasma concentration, in the hope of finding a simple indirect method of calculation of the plasma concentration from that in the urine, such as was found in the case of mepacrine (Army Malaria Research Unit, 1945). It was shown that the urinary concentration of mepacrine was a direct function of the urinary ammonia content and the plasma mepacrine concentration. A long series of experiments, in which the urinary

ammonia content was varied and samples of plasma and urine taken and estimated simultaneously, showed that in the case of Paludrine no such relationship exists. It was found, also, that the use of ammonia oxalate as an anticoagulant did not affect the distribution of Paludrine between the cellular elements and plasma, as it does in the case of mepacrine.

The relation between the 24-hour urinary output of Paludrine and the minimal plasma concentrations obtaining during the same period in a patient on a 12-hourly dosage régime has not been clearly established. Some degree of correlation exists between plasma concentration and urinary output over 24 hours, as can be seen by inspection of fig. 2, but we have not been able to establish any simple relation between the plasma concentration and the urinary output or concentration. Investigation of the urinary elimination of the drug after a single dose of Paludrine has also failed to reveal any close relation between the plasma concentrations reached and the urinary output or concentration.

THE EXCRETION OF PALUDRINE IN THE FAECES

Total balance experiments were carried out in patients and volunteers in which plasma concentrations and both urinary and faecal excretion of Paludrine were measured over periods of time following the administration of both single doses and full therapeutic dosage régimes of the drug. Examples of the results obtained are set out in Tables V, VI and VII.

TABLE V
Excretion of Paludrine in urine and faeces following a single oral dose of 100 mgm.

Time	Daily Paludrine excretion in	
	Faeces	Urine
1st 24 hours	0.26 mgm.	31.96 mgm.
2nd "	4.95 mgm.	4.38 mgm.
3rd "	3.30 mgm.	1.11 mgm.
4th "	0.45 mgm.	0.53 mgm.
Total ...	8.96 mgm., or 9.0 per cent. of intake	37.98 mgm., or 38.0 per cent. of intake

TABLE VI
Excretion of Paludrine in urine and faeces following a single oral dose of 500 mgm.

Time	Daily Paludrine excretion in	
	Faeces	Urine
1st 24 hours	30.6 mgm.	190.45 mgm.
2nd "	22.4 mgm.	54.20 mgm.
3rd "	6.55 mgm.	10.70 mgm.
4th "	1.07 mgm.	9.35 mgm.
5th "	1.33 mgm.	4.30 mgm.
6th "	0.62 mgm.	2.35 mgm.
7th "	—	1.05 mgm.
Total ...	62.57 mgm., or 12.5 per cent. of intake	272.40 mgm., or 54.5 per cent. of intake

TABLE VII
Excretion of Paludrine in urine and faeces in a patient receiving 500 mgm.
b.d for 14 days

Time	Daily Paludrine excretion in	
	Faeces	Urine
1st 24 hours	224 mgm.	174 mgm.
2nd "		—
3rd "		527 mgm.
4th "		605 mgm.
5th "	141 mgm.	662 mgm.
6th "		528 mgm.
7th "		347 mgm.
8th "		474 mgm.
9th "	153 mgm.	484 mgm.
10th "		550 mgm.
11th "		396 mgm.
12th "		294 mgm.
13th "	193 mgm.	470 mgm.
14th "		403 mgm.
15th "		211 mgm.
16th "		63 mgm.
17th "	66 mgm.	24 mgm.
18th "	81 mgm.	9 mgm.
19th "	14 mgm.	3 mgm.
20th "	2 mgm.	2 mgm.
21st "	1 mgm.	
Total ...	1,021* mgm., or 7.3 per cent. of intake	6,226* mgm., or 44.5 per cent. of intake

* No allowance has been made for the day on which the sample was not sent in for analysis.

It will be seen from the tables that the percentage of the total dosage of Paludrine excreted in the faeces is of the same order after single doses of 500 and 100 mgm. as it is after repeated dosage: it accounts for about 10 per cent. of the ingested drug. The fact that, over all dosage régimes investigated, the percentage excreted in the faeces is about the same suggests that the drug is probably almost completely absorbed from the gut. If this were not so, the percentage loss would increase with the dosage. It is thus likely that the drug recovered from the faeces has been absorbed and excreted into the intestine. Such excretion in the bile occurs with certain other antimalarial drugs, such as mepacrine (Hecht, 1933; Annegers *et al.*, 1943; Army Malaria Research Unit, 1946*a*), quinine (Bernardbeig and Caujolle, 1935), and 3349 (Spinks and Tottey, 1946*b*). In the case of mepacrine and 3349, however, the faecal excretion accounts for a higher proportion of the total excretion than does urinary excretion. In the case of Paludrine this state of affairs is reversed.

SUMMARY

1. The plasma concentrations of Paludrine observed following therapeutic doses of the drug both orally and by the intravenous route are given.
2. Blood concentrations of Paludrine are normally about three times those found in the plasma.
3. Results of preliminary experiments indicate that about 75 per cent. of the plasma Paludrine content is protein-bound.
4. Forty to sixty per cent. of the drug administered is excreted by the kidneys.
5. About 10 per cent. of the drug administered is excreted in the stools.

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